HEALTH CENTER LIBRARY

THE TOHOKU JOURNAL OF EXPERIMENTAL MEDICINE

Incorporating

ARBEITEN AUS DEM ANATOMISCHEN INSTITUT
DER KAISERLICH-JAPANISCHEN
UNIVERSITÄT ZU SENDAI
AND
MITTEILUNGEN ÜBER ALLGEMEINE PATHOLOGIE
UND PATHOLOGISCHE ANATOMIE

STATE UNIVERSITY SEP 24 1959

Vol. 69, No. 4, April 25, 1959

EDITORIAL BOARD
T. KATO H. MASAMUNE K. MOTOKAWA M. WADA

WITH COLLABORATION OF
Y. AKAGI, K. AKAZAKI, T. EBINA, T. FUJITA, Y. HAYASHI, S. IINO,
T. ISHIBASHI, T. ISHIKAWA, M. ITO, K. IWATSUKI, S.-T. KATSURA,
N. KIRISAWA, Y. KOGA, S. KONDO, T. KUMAGAI, T. KUROKAWA,
M. KUROYA, K. KUSHIMA, T. MURAKAMI, M. MUTO, F. NAKAZAWA,
S. NASU, S. OSATO, T. SANO, A. SATO, S. SATO, T. SHIMAUCHI,
M. SEGI, H. SETO, T. SHINODA, SH. SUGIMURA, T. SUGIYAMA,
N. SUWA, T. SUZUKI, E. TAKAHASHI, Y. TAKAHASHI, M. TERASAKA,
T. TORIGAI, Y. TUIKI, R. URA, S. YAGI, S. YAMAGATA

PUBLISHED BY
THE TOHOKU UNIVERSITY
SENDAI, JAPAN

SOLD BY
MARUZEN COMPANY, LTD., TOKYO
CHARLES E. TUTTLE COMPANY, RUTLAND, VERMONT, U. S. A.

The Tohoku Journal of Experimental Medicine is an official organ of the Tohoku University for the publication of records of original research in all branches of experimental medicine, but accepts also papers from sources other than the University, domestic as well as overseas, under certain limitations, given below.

Two to three volumes, each containing four numbers, are issued a year. Each volume will consist of approximately 400 pages.

The articles are written in English, French or German.

The subscription price is $5.00, foreign $5.50, per volume, postpaid, and should be sent to the MARUZEN CO., LTD., Tokyo or CHARLES E. TUTTLE CO. RUTLAND, VERMONT, U.S.A.

Papers and correspondence regarding exchange of publications, etc. should be addressed to the Medical Library, Tohoku University, Kita-yoban-tyo, Sendai.

Notice to Contributors

Papers will not be accepted for simultaneous publication or those which have been previously published in the periodicals written in English, French, German or Italian. All articles from Laboratories must be approved by the director of the originating laboratory. Contributions should be ordinarily limited in length to 10 printed pages. Every paper must be accompanied by a brief summary.

The manuscripts should be typewritten in double spacing, and carefully revised before submission to the Editor. The original, not carbon, copy should be sent. Bibliographies should give the name of the author, the name of journal or book (with edition & place of publishing), the year, the volume and the page.

Figures should be ready for photographic reproduction, diagrams should be drawn in Indian ink and plain white or fine blue-lined paper only should be employed ; letters, numbers etc. should be written in pencil. The reduction desired should be clearly indicated on the margin of the drawing. The Journal cannot accept illustrations which when reproduced for printing exceed the size of a Journal page. Authors should indicate on the manuscript the approximate position of the text figures and tables.

All the drawings and tables should be marked with the author's name. They should be packed plate, carefully protected from injury, and insured.

Proofs will not be submitted to authors overseas unless they express a desire to see them, or unless the editors think it necessary. Overseas authors are therefore particularly reminded of the importance of preparing their manuscripts in such a way that errors need not arise.

Authors receive 100 reprints of articles at cost.

The Tohoku Journal of Experimental Medicine, Vol. 69, No. 4, 1959

Three Types of Behavior of Tuberculin-Polysaccharide in Blood for the Evaluation of the Activity of Tuberculosis in Infancy and Childhood

Studies on Tuberculosis in Infancy and Childhood. 5th Report

By

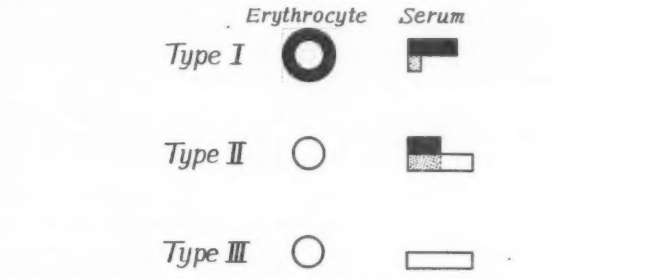
Tsuneo Arakawa, Mizuo Oota and **Yuji Sato**
(荒川 雅男) (太田 瑞夫) (佐藤 雄治)

From the Department of Pediatrics, Faculty of Medicine, Hirosaki University, Hirosaki; Director: Prof. Ts. Arakawa

(Received for publication, January 22, 1959)

Basing upon our own experiments[1)2)3)] respecting the tuberculin-polysaccharide in the blood, we are of the opinion that the activity of the tuberculous foci in infancy and childhood will be evaluated by examining the behavior of the tuberculin-polysaccharide in the blood. As is shown in Fig. 1 and Table I, the occurrence of three types (Types I, II and III) of the behavior of tuberculin-polysaccharide in the blood will be seen.

Fig. 1. Schema of the Relation between Tuberculin-polysaccharide and the Antibody in the Blood.



Erythrocytes sensitized with tuberculin-polysaccharide.

Erythrocytes not sensitized with tuberculin-polysaccharide.

Tuberculin-polysaccharide.

Antibody against tuberculin-polysaccharide, which is able to be detected by the Middlebrook-Dubos reaction.

Antibody against tuberculin-polysaccharide, which is combined with tuberculin-polysaccharide, consequently unable to be detected by the Middlebrook-Dubos reaction.

TABLE I

Illustration of Types I, II and III in Tuberculous Cases

	The erythrocytes sensitized with tuberculin-polysaccharide in the circulating blood	The tuberculin-polysaccharide in serum	The antibody against tuberculin-polysaccharide in serum, which is able to be detected by the Middlebrook-Dubos reaction	Clinical significance
Type I	+	+	−	active tuberculosis
Type II	−	+	+	active tuberculosis
Type III	−	−	+	Not active

Type I :—Tuberculin-polysaccharide is identified as being adsorbed to the erythrocytes in the circulating blood, and a part of the tuberculin-polysaccharide is existing in the serum, while the antibody against the tuberculin-polysaccharide in the serum is unable to be detected by means of the Middlebrook-Dubos reaction[1)3)].

Type II :—Tuberculin-polysaccharide is identified as being combined with the antibody in the serum[4)], but the erythrocytes sensitized with the tuberculin-polysaccharide are not found in the circulating blood, and the antibody in the serum is able to be detected by the Middlebrook-Dubos reaction.

Type III :—Tuberculin-polysaccharide is not identified in the serum, and erythrocytes are free from the tuberculin-polysaccharide, and the antibody in the serum is detected by the Middlebrook-Dubos reaction.

From the clinical point of view, these three types occurred generally in the following conditions respectively :—

Type I :—Early stage of tuberculous infection in infancy and childhood, in which the antibody usually would be poorly formed.

Type II :—1) Some of simple tuberculin converters, 2) an exogenous infection of tuberculosis upon the individual who had BCG-vaccination, and 3) the activation of tuberculous foci by an intercurrent illness.

Type III :—The cases in which the tuberculous foci were no more active.

The incidence of these three types was examined in 235 patients who were under 13 years of age and who had only banal complaints such as anorexia, pallor, fever of low grade, diarrhea and so on. They might or might not have been tuberculously infected, and 140 cases of them had been treated or under way of treatment by some antituberculous drug or other.

As is given in Table II, Type I was found only in the cases below 4 years of age and the incidence of Type III was higher than that of Type II

TABLE II

Incidence of Types I, II and III in 235 Cases with Banal Complaints

	No. of the cases examined	No. of cases		No. of cases which had been given chemotherapy continuously or for some time past
		Under 4 years of age	5–13 years of age	
Type I	3	3	0	0
Type II	76	40	36	29
Type III	156	22	134	111
Total	235			140

among the cases which had been chemotherapeutically treated for some time past or were under the way of chemotherapy (Cf. Table II).

Our clinical examination and type examination have shown that, in infancy and childhood, the change from Type I to Type III through Type II or the change from Type II to Type III, was going hand in hand with an improvement of clinical symptoms by an effective treatment[5].

So it has been concluded that, when Type I or Type II is identified in a tuberculous case in infancy and childhood, tuberculous foci will be accepted as *active*.

CONCLUSIONS

The activity diagnosis of tuberculous infection in infancy and childhood is described.

Three types (Types I, II and III) of behavior of the tuberculin-polysaccharide in blood are found, and when Type I or Type II is detected in a tuberculous case in infancy and childhood, the tuberculous foci will be active.

References

1) 2) 3) Arakawa, Oota & Sato, Tohoku J. Exp. Med., 1958, **68**, 93, 99, 105.

4) Arakawa, Oota & Sato, Approximate Estimation of Tuberculin-polysaccharide in serum. To be published in this Journal.

5) Arakawa, Oota & Sato, Tuberculin-polysaccharide in the Blood of Tuberculous Infant and Children: Influence of Chemotherapy upon Same. To be published in this Journal.

The Tohoku Journal of Experimental Medicine, Vol. 69, No. 4, 1959

Effect of Adrenalectomy on the Excitability of Plantar Sweat Glands in Albino Rats

By

Kazukuni Yamashita

(山下 一邦)

From the Department of Physiology, Nagasaki University School of Medicine, Nagasaki

(Received for publication, March 2, 1959)

The present study was undertaken in order to show how the removal of adrenal glands affects the excitability of plantar sweat glands in rats. In so far as we know, no evidence has been provided on this point.

Experiments were performed on adult male rats. The excitability of the plantar sweat glands was measured by determining the minimum effective concentration of mecholyl for causing local sweating. After excluding the spontaneous sweating by ether anesthesia, mecholyl chloride (Merck) dissolved in 0.9% NaCl solution was injected intradermally, and sweat secretion was visualized by use of the iodine starch method of Wada and Takagaki.[1] Both adrenal glands were removed at one time by the lumbar approach.

All the animals died within 6–12 days after adrenalectomy. The excitability of the plantar sweat glands remained unaltered for several days after adrenalectomy. The minimum effective concentration was measured to be 10^{-8}–10^{-9} before as well as after adrenalectomy. Then, in 8 out of 10 animals, sudden drops of the excitability were observed. The minimum effective concentration increased up to 10^{-5}–10^{-6}. The animals died on the following days. In the 2 others, however, no significant alteration in the excitability was observed throughout the experiment.

The author wishes to express his indebtedness to Prof. T. Suzuki for helpful suggestions.

References: 1) Wada, M. & Takagaki, T., Tohoku J. Exp. Med., 1948, **49**, 284.

The Tohoku Journal of Experimental Medicine, Vol. 69, No. 4, 1959

Diets and Progress of Alloxan Diabetes

By

Tatuo Saito, Akira Wakui and **Akira Ohneda**
(斉藤達雄) (涌井昭) (大根田昭)

From the Medical Clinic of Prof. T. Kurokawa, Tohoku University, Sendai

(Received for publication, January 22, 1959)

Although recently various orally administrated drugs have been used in the treatment of diabetic patients, the principle of treatment of diabetes mellitus consists of diet and insulin.

Various dietary formula have been proposed. In 1936, Prof. Yamakawa proposed a system of high-carbohydrate and low-fat diet for diabetic patients[1)2)]. Since then, diabetic patients have been treated with this dietary formula in our Clinic. In general, the diet system for diabetic patients throughout the world is, to our impression, apparently approximating those of our system.

On the other hand, numerous methods have been available for producing a permanent diabetes mellitus. There are several already known dietary stuffs such as uric acid[3)], glucose[4)5)], acetoacetic acid[6)] and beta-hydroxybutyric acid[7)] to produce an experimental diabetes mellitus.

If, as we already showed[8)], high-protein high-fat diet had the potency of development of an experimental diabetes mellitus, this diet might be harmful in the treatment of diabetic rats.

The present paper deals with the effect of different diets on the progress of diabetic rats induced by alloxan administration.

Experimental

Material and Method

Twenty five normal male rats of the Wistar strain were used. They were divided into five groups and fed with different diets during a whole experimental period as shown in Table I.

After fasting 12 hours, animals were subjected to glucose tolerance test, in which glucose was given orally in amount of 2.0 gm. per kg. body weight.

Blood was taken from the tail vein and the blood sugar level was estimated by the micromethod of Fujita and Iwatake.[9)] Then 5% aqueous solution of alloxan was injected to the femoral veins of all animals in amount

TABLE I

Composition of Diet

Figures express weight proportion to total of 100, excepting those showing caloric values.

Component	Diet Group				
	I Standard	II High-protein	III High-fat	IV High-protein high-fat (isocaloric)*	V High-protein high-fat (high-caloric)
Carbohydrate	70	61	46	10	35
Protein	12	22	12	36	25
Fat	8	7	32	44	34
Vitamin and salt	10	10	10	10	10
Calories per day	82	92	107	82	105

Note :—Carbohydrate, Irish potato flour. Protein, casein. Fat, lard. Vitamin and salt, Ebios, McCollums salt and cod liver oil in proportions of 4:4:2 by weight.

* With equal calories to the standard diet.

of 30 mg. per kg. body weight several days after the first glucose tolerance test.

Glucose tolerance tests were again carried out two and four weeks after alloxan administration.

All animals were sacrificed for the pathohistological studies about forty days after alloxan administration.

The free acetone value was estimated by Shimazono's modified[10] method of Greenberg and Lester in about half of them.

Results

Glucose tolerance test before alloxan administration

The results of glucose tolerance test is shown in Figure 1. The mean value of blood sugar was 114 mg% before glucose administration, 117 mg% in its peak and 106 mg% after three hours, respectively. The data are summarized in Table II.

Glucose tolerance test two weeks after alloxan administration

In all groups decreased glucose tolerance was observed and there were no difinite differences between the gorups. The glucose tolerance, however, was particularly markedly decreased in the group of high-protein high-fat high-caloric diet (shown in Tables III to VII).

Glucose tolerance test four weeks after alloxan administration

Perceptible improvement was observed in the groups of standard diet and high-fat diet four weeks after alloxan administration. There were no

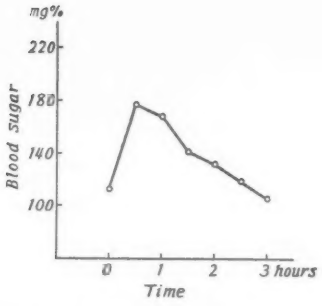


Fig. 1. Glucose tolerance test of normal rats.

TABLE II

Average Glucose Tolerance Test of Normal Rats

Time (hrs.)	0	0.5	1.0	1.5	2.0	2.5	3.0
Blood sugar (mg%)	114	177	167	142	132	120	106
Standard deviation (mg%)	18	18	20	20	13	13	19

TABLE III

Glucose Tolerance Test of the Group of Standard Diet

No. of rats	Blood sugar level (mg%)							Urine sugar
	Before glucose loading	Time (hrs.) after glucose loading						
		0.5	1.0	1.5	2.0	2.5	3.0	
a) Two weeks after alloxan administration								
1	201	334	431	395	271	264	264	+
2	170	289	261	278	239	146	135	+
3	94	198	224	208	200	132	87	+
4	142	182	149	121	97	111	114	−
b) Four weeks after alloxan administration								
1	156	242	322	312	294	292	260	+
2	114	236	176	171	156	148	148	−
3	91	177	214	177	180	142	90	−
4	122	174	170	194	188	128	126	−

appreciable changes in the group of high-protein diet, while diabetes was markedly aggravated in all animals of groups of high-protein high-fat,

TABLE IV

Glucose Tolerance Test of the Group of High-Protein Diet

No. of rats	Blood sugar level (mg%)							Urine sugar
	Before glucose loading	Time (hrs.) after glucose loading						
		0.5	1.0	1.5	2.0	2.5	3.0	
a) Two weeks after alloxan administration								
7	121	198	201	224	201	194	180	+
8	161	326	348	294	224	180	146	+
10	132	194	215	153	146	90	146	−
b) Four weeks after alloxan administration								
7	122	204	192	184	174	148	162	−
8	138	208	198	208	192	184	170	−
10	138	198	354	374	278	264	226	+

TABLE V

Glucose Tolerance Test of the Group of High-Fat Diet

No. of rats	Blood sugar level (mg%)							Urine sugar
	Before glucose loading	Time (hrs.) after glucose loading						
		0.5	1.0	1.5	2.0	2.5	3.0	
a) Two weeks after alloxan administration								
11	146	224	246	242	222	194	194	+
12	235	264	330	326	270	256	240	+
13	201	268	208	191	194	160	208	+
14	94	271	201	205	201	181	177	+
15	100	242	233	285	222	174	128	+
b) Four weeks after alloxan administration								
11	118	239	222	184	167	—	153	+
12	138	222	192	184	176	166	162	±
13	132	156	184	200	192	166	192	+
14	114	194	184	176	166	156	156	−
15	148	208	212	267	194	170	166	+

especially high-caloric diet (shown in Tables III to VII and Figs. 2 to 7). Therefore, the carbohydrate assimilations were divided into three groups, improved, deteriorated and unchanged in comparison with the result obtained in two weeks after alloxan administration (shown in Table VIII).

The lebel of free acetone in the blood was elevated in the group of

TABLE VI

Glucose Tolerance Test of the Group of High-Protein High-Fat Isocaloric Diet

No. of rats	Blood sugar level (mg%)							Urine sugar
	Before glucose loading	Time (hrs.) after glucose loading						
		0.5	1.0	1.5	2.0	2.5	3.0	
a) Two weeks after alloxan administration								
16	100	247	236	261	254	254	201	+
17	177	219	254	264	242	257	187	+
18	146	337	355	295	309	337	302	+
19	127	264	292	280	180	156	146	+
20	112	246	302	256	246	228	201	+
b) Four weeks after alloxan administration								
16	128	148	166	212	184	184	167	−
17	160	200	236	278	224	222	217	+
18	160	260	326	344	306	278	270	+
20	101	222	321	312	292	214	174	+

TABLE VII

Glucose Tolerance Test of the Group of High-Protein High-Fat High-Caloric Diet

No. of rats	Blood sugar level (mg%)							Urine sugar
	Before glucose loading	Time (hrs.) after glucose loading						
		0.5	1.0	1.5	2.0	2.5	3.0	
a) Two weeks after alloxan administration								
21	191	274	289	351	317	306	278	+
22	122	264	361	308	290	212	167	+
23	94	226	239	181	167	149	163	+
24	153	194	201	181	153	149	130	−
25	146	187	205	292	302	235	213	+
b) Four weeks after alloxan administration								
21	170	234	306	360	348	282	234	+
22	191	274	289	351	317	306	278	+
23	208	306	368	309	233	233	236	+
24	198	236	236	215	202	215	191	+
25	180	208	330	302	294	266	233	+

Fig. 2

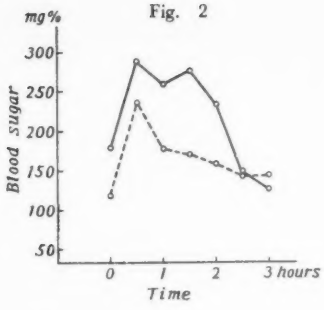


Fig. 3

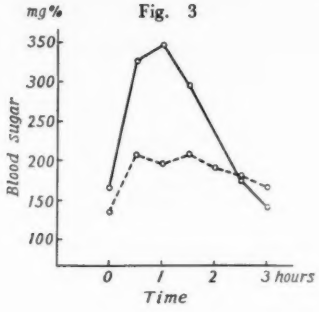


Fig. 4

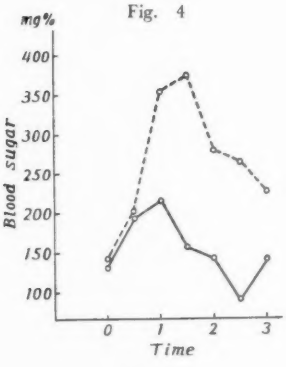


Fig. 5

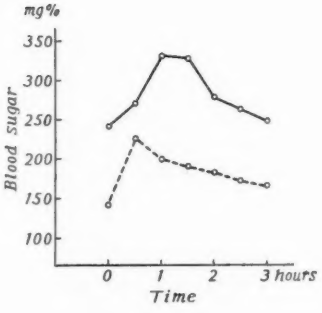


Fig. 6

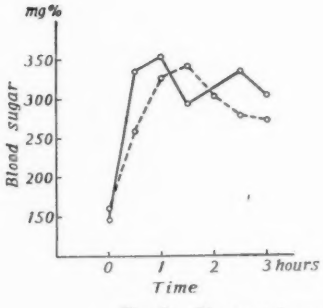


Fig. 7

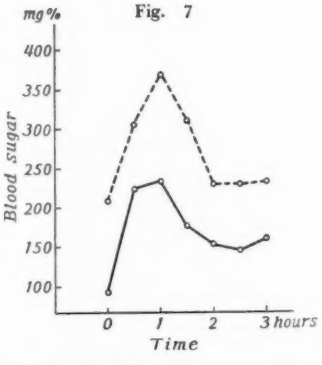


Fig. 2. Glucose tolerance test of No. 2 fed with standard diet.

Fig. 3. Glucose tolerance test of No. 8 fed with high-protein diet.

Fig. 4. Glucose tolerance test of No. 10 fed with high-protein diet.

Fig. 5. Glucose tolerance test of No. 12 fed with high-fat diet.

Fig. 6. Glucose tolerance test of No. 18 fed with high-protein high-fat isocaloric diet.

Fig. 7. Glucose tolerance test of No. 23 fed with high-protein high-fat high-caloric diet.

The solid and broken lines in Figures 2 to 7 represent the glucose tolerance curves two and four weeks after alloxan administration respectively.

TABLE VIII

The Effect of Diets upon the Course of Alloxan Diabetes in Rats

Diets	Course			
	Improved	Unchanged	Deteriorated	Death
Standard diet	2	2	0	1 (Not obtainable)
High-protein diet	2	0	1	2
High-fat diet	4	1	0	0
High-protein high-fat isocaloric diet	1	3	0	1
High-protein high-fat high-caloric diet	0	1	4	0

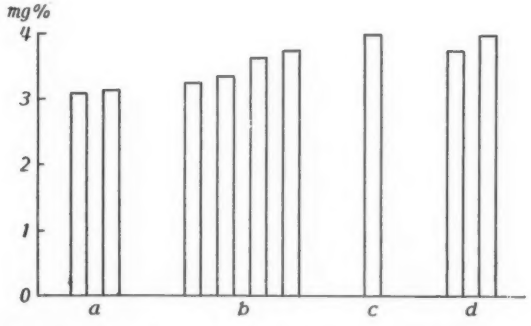


Fig. 8. Blood free acetone. a...the group of standard diet. b...the group of high-fat diet. c...the group of high-protein high-fat isocaloric diet. d...the group of high-protein high-fat high-caloric diet.

high-protein high- fat high-caloric diet (shown in Fig. 8).

Pathohistological findings

The pathohistological changes in the pancreas, adrenal glands and anterior pituitary lobe were most prominent in the group of high-protein high-fat high-caloric diet, as compared with the group of standard diet.

a) Langerhans' islets of the pancreas. The remaining beta-cells were found to be hypertrophied, and a picture of regeneration of the alpha-cells was observed in the marginal parts of the islet cells in the group of standard diet (shown in Fig. 9). The collapse of beta-cells was widespread and decrease in number of beta-cells was notable in the groups of high-protein high-fat diet, both in the isocaloric and high-caloric groups. No tendency of regeneration were seen in these groups (Figure 10).

b) Adrenal glands. Generally speaking, the medulla was chromoafinitive and showed no significant changes, while in the cortex the follow-

Fig. 9

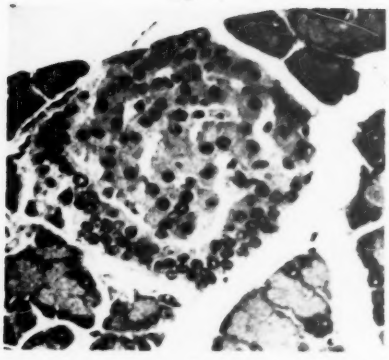


Fig. 10

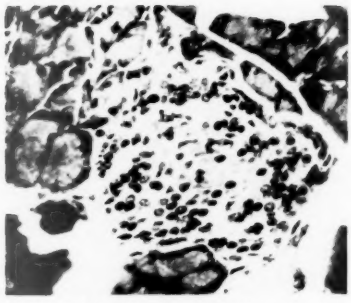


Fig. 9. Regeneration of Langerhans' islet. No. 3 fed with standard diet. Gomori's chrome alum hematoxylin stain.

Fig. 10. Marked collaps of Langerhans' islet. No. 21 fed with high-protein high-fat high-caloric diet. Gomori's chrome alum hematoxylin stain.

ing numerous changes were revealed. The fascicular layer was thick, swollen and transparent and the distinction between the light and dark cells in it was very perceptible in the group of high-protein high-fat diet in which the glucose tolerance was definitely impaired, as compared with that in the group of standard diet in which the improvement of the glucose tolerance was shown (Figs. 11 to 14).

c) The anterior pituitary lobe. Some changes in this organ were observed in these groups. Eosinophilic cells showed no increase in number but were slightly degranulated and swollen in the groups of standard, high-protein and high-fat diet in which the carbohydrate metabolism were observed to be improved (Fig. 15).

The changes of the eosinophilic cells were somewhat milder but the small sized basophilic cells were rather increased in number in one rat in the group of high-protein diet and almost all the animals in the group of high-protein high-fat diet (Fig. 16). It is noted that the severity of these histological changes was proportional to that of disturbance in the carbohydrate metabolism as evaluated by the results of glucose tolerance tests.

No perceptible changes were observed in the liver and kidneys.

Discussion

It has been shown that a high-protein high-fat diet in normal rats contains a diabetogenic character. Therefore a hypothesis was proposed that, if diabetic rats induced by alloxan administration are fed with high-protein high-fat diet, diabetic symptoms may become deteriorated.

The present data showed that standard group, of which a diet system

Fig. 11

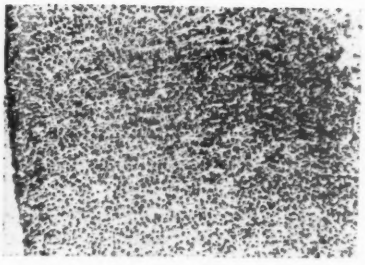


Fig. 12

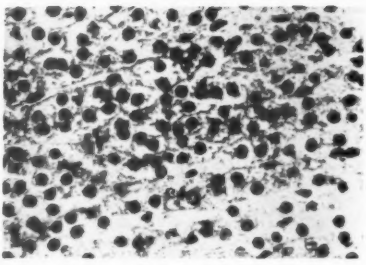


Fig. 13

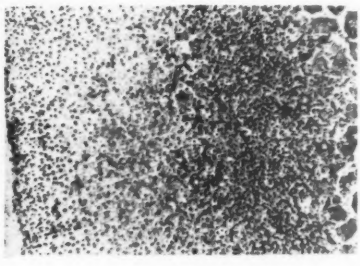


Fig. 14

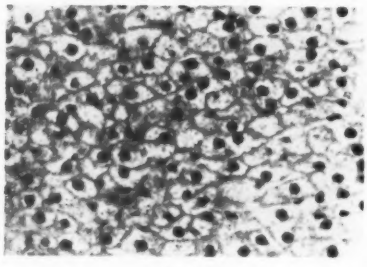


Fig. 15

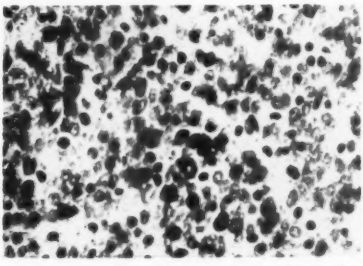


Fig. 16

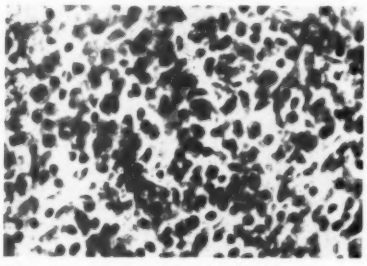


Fig. 11. Adrenal gland of the animal in the group of standard diet. Hematoxylin-eosin stain.

Fig. 12. High power view of Fig. 11.

Fig. 13. Adrenal gland of No. 23 fed with high-Protein high-fat high-caloric diet. Thick, swollen and transparent fascicular layer shows the distinction between the light and dark cells. Hematoxylin-eosin stain.

Fig. 14. High power view of Fig. 13.

Fig. 15. Anterior pituitary lobe, showing slightly degranulated eosinophilic cells. No. 7 fed with high-protein diet. Masson-Goldner's stain.

Fig. 16. Anterior pituitary lobe, showing the increase in number of small sized basophilic cells. No. 23 fed with high-protein high-fat high-caloric diet. Masson-Goldner's stain.

is similar to our clinical dietary one brought out by Prof. Yamakawa and Kurokawa, was improved in the progress of experimental diabetes mellitus

induced by alloxan administration.

In a number of clinical observations on the effect of diet for the treatment of diabetic patients it has been recognized that high-carbohydrate low-fat diet or dietary system containing the three main food stuffs in well balanced propotion is superior to other dietary systems.

It is well known that an increase in fat content will ameliorate the diabetic symptoms[11] as well as the acetylation values[12] in alloxan diabetic rats.

Houssay and Martinez[13], however, reported that the toxic and diabetogenic action of alloxan increased in rats fed with a high lard diet. They observed on the toxic action of alloxan but not on the effect along the progress of alloxan diabetes.

In our experiments, a diet with high-fat content appeared to improve the glucose tolerance and the histological findings also showed signs of regeneration of beta-cells in Langerhans' islets in the group of high-fat diet. Our results come to an agreement with that shown by Burn.[11]

On the other hand, there were both improvement and deteriolation in the glucose tolerance in the group of high-protein diet. Protein, therefore, in the content allowed in the current diet system of diabetic animals, seems to have little effect on the carbohydrate metabolism.

Several authors showed different results on the effects of protein in the dietary treatment for diabetic patients[14][15][16]. Thus a significance of protein in diet is left to be studied furthermore.

However, there were aggravations both in the glucose tolerance and histological findings of Langerhans' islets in the animals fed with high-protein high-fat diet. Such a diet, therefore, should be branded as harmful to the treatment of diabetic animals.

Kotake[17] recently reported that a high-protein high-fat diet will produce a state of vitamin B_6 deficiency in rats and xanturenic acid derived from tryptophan in urine, which might be diabetogenic.

We, however, do not entirely agree with the opinion of single diabetogenic factor, such as xanturenic acid, since glucose tolerance was decreased and the histological studies including pancreas, adrenal glands and pituitary showed the pluriglandular changes in the normal rats fed with high-protein high-fat diet.

Of course, beta-cells of Langerhans' islets were extremely collapsed and moreover the thickening of the fascicular layer in the adrenal glands and the increase in number of small sized basophilic cells in the anterior pituitary lobe were observed in the diabetic animals which showed the marked impairment in glucose tolerance test.

Therefore, we would adovocate that the pituitary adrenal system plays a part in the progress of experimental diabetes mellitus induced by various

diets. It has been already reported that the administration of the extract of pituitary[18] or cortical steroids[19] could produce experimental diabetes mellitus.

Summary

We studied the effects of various diets for the treatment of diabetic rats induced by alloxan administration.

The progress of diabetic symptoms was improved favorably in the group of standard diet but deteriorated both in the glucose tolerance and histological studies in the group of high-protein high-fat diet.

The changes of the adrenal cortex such as the thickening of the fascicular layer and the distinction between the light and dark cells in it and that of the pituitary such as the increase in number of small sized basophilic cells shown in the rats fed with high-protein high-fat diet were parallel with a decreased glucose tolerance.

Therefore, the dietary system containing three main food stuffs in well-balanced propotion as our standard diet is superior to others in the treatment of diabetic animals induced by alloxan administration.

References

1) Yamakawa & Yamauchi, Shindan To Chiryo (Jap.), 1936, **23**, 619.
2) Yamauchi, Tohoku J. Exper. Med., 1938, **32**, 268, 289.
3) Griffiths, J. Biol. Chem., 1948, **172**, 853.
4) Dohan & Lukens, Science, 1947, **105**, 183.
5) Yoshikawa, Tohoku Igaku Zasshi (Jap.), 1954, **49**, 25.
6) Nath & Chakrabati, Proc. Soc. Exper. Biol. Med., 1950, **75**, 326.
7) Nath & Brahmachari, Nature, 1944, **154**, 487.
8) Saito, J. Jap. Soc. Int. Med. (Jap.), 1958, **47**, 864.
9) Fujita & Iwatake, Biochem. Zschr., 1931, **242**, 43.
10) Koide, Mukaiyama & Morita, Seikagaku (Jap.), 1953, **25**, 306.
11) Burn, Lewis & Kelsey, Brit. Med. J., 1944, **2**, 752.
12) Charalampous & Hegsted, J. Biol. Chem., 1949, **180**, 623.
13) Houssay & Martinez, Science, 1947, **105**, 548.
14) Vartiainen & Apajalahti, Ann. Med. Intern. Fenn., 1955, **44**, 95.
15) Cochrane, Payne, Simpkiss & Woolf, J. Clin. Invest., 1956, **35**, 411.
16) Srinivasan, Lancet, 1957, **2**, 319.
17) Kotake & Tani, J. Bioch. (Japan), 1953, **40**, 295.
18) Young, Lancet, 1937, **233**, 372.
19) Ingle, Sheppard, Evans & Kuizenga, Endocrinology, 1945, **37**, 341.

The Tohoku Journal of Experimental Medicine, Vol. 69, No. 4, 1959

Toxic Substances in Cancer

VIII. Effect of the Aqueous Extract of Gastric Carcinoma Tissue on the Growth of Paramecium*

By

Mutsuhiko Yamaguchi

(山口睦彦)

From Prof. M. Muto's Surgical Clinic, Tohoku University, Sendai

(Received for publication, January 23, 1959)

In a series of the studies on the toxic substances in cancer, Oh-Uti and his collaborators have demonstrated the substances inhibiting the movement of *Paramecium* in the extract of gastric carcinoma tissue[1)2)] as well as in the gastric juice of patients with gastric carcinoma[3)].

In the present work, the substances derived from the tissue of gastric carcinoma were tested for effects on the growth of *Paramecium*.

I express my sincere thanks to the late Professor Gunji Tomita of the Biological Institute of our university and to Assistant Professor Sadaaki Koizumi of the Biological Institute of the Iwate University for their helpful advice throughout the work. Thanks are also due to the Surgical Department of the Sendai National Hospital (Director: Dr. K. Kikuchi) from which a part of the amaterial was supplied.

Experimental

Method for Cultivation

1) *Paramecium*

The animal used in the present work was *Paramecium caudatum*, stock K_{A6} kept in the Biological Institute of our university. This was the same stock as used in the previous investigations.[1)2)3)]

2) Culture medium

The culture medium used for all the experiments was a baked lettuce infusion inoculated with *Aerobacter aerogenes* which was prepared as follows according to Koizumi *et al.*[4)]

The separated clean leaves of a head of lettuce were washed with water, dried in an oven until they turned brown, and then ground to powder in a mortar. The dried lettuce powder thus obtained was then boiled with water for 10 to 15 minutes (1.5 g. baked lettuce powder was added to each liter of

* Oh-Uti and his coworkers' "Biochemical Studies on Cancer (28th Report)".

water) and the mixture was filtered. To the filtrate was added about 0.2 g./l. of calcium carbonate and the solution was allowed to stand in an ice box after being made sterile by means of autoclaving for 20 minutes at 120°C.

When ready for use, the lettuce infusion was inoculated with *Aerobacter aerogenes* which had been cultivated successively on bouillon-agar slants, and then incubated at 26.0±0.5°C. for 18 to 24 hours. The pH of the medium was finally adjusted to 7.0 with sterile suspension of calcium carbonate.

For preparing the culture medium, spring water was used after being filtered, boiled and then allowed to cool, which was, and will be, referred to simply as water.

3) Culture method

One hundred to 300 sterilized test tubes (1.5 cm. in diameter and 11 cm. in height), each of which had been filled with 10.0 cc. of the culture medium, were used for carrying every series of the experiment.

First, 50 individuals of *Paramecium* were put into each test tube in the manner as follows. A suspension of *Paramecium* in a test tube was kept straight for a while. Then a layer just below the surface, where the organisms were aggregated because of negative geotropism, was transferred by a sterilized pipette into another sterilized test tube filled with sterile water. The mass of *Paramecium*, both condensed enough and freed from the culture medium by such manipulations repeated twice more, was kept in fast for 24 hours. It was then diluted with sterile water to make a suspension containing 50 individuals per one drop of the standard pipette, which had been designed in the manner that 1 cc. of a suspension should make accurately 50 drops. The suspension thus prepared was well mixed, taken in the standard pipette and transferred quickly to the test tubes for cultivation, one drop per tube.

The tubes were then left to stand at 26.0±0.5°C. after being stoppered tightly.

At the given time, five of the test tubes were taken at random and shaken well to sufficiently mix the contents. Then, using the above-mentioned standard pipette, 10 to 25 drops of the suspension from each of the test tubes were put on a slide depression and the number of *Paramecia* contained was counted under a microscope, from which was calculated the total number of the living individuals in the test tube. The average number of the living *Paramecia* from the five test tubes was regarded to present the number of *Paramecia* at the time when the measurement was performed.

In experiments for observing the standard growth, the calculation of number of living *Paramecia* was carried out every 24 hours. Method for the cultivation with the test materials will be stated later.

Standard Growth

1) Curve of the standard growth

For the purpose of obtaining the standard feature of the growth of *Paramecium* under the above condition, five series of the experiment were performed without adding any of the test materials.

Results are given in Table I. The number of *Paramecia* cultivated from 50 individuals was 90 to 150, 120 on an average after 24 hours; 830 to 1250, 1000 after 48 hours; 1840 to 3160, 2590 after 72 hours; 2920 to 3720, 3360 after 96 hours; 3660 to 4340, 3980 after 120 hours; 3440 to 4600, 4060 after 144 hours and 4120 to 5300, 4500 after 168 hours. Thus the curves of the growth (Fig. 1) are sigmoid in form, having a strong resemblance to those obtained by Koizumi *et al.*[4]

TABLE I

Number of Paramecia in the Standard Growth of 50 Organisms

Time (hours)	24	48	72	96	120	144	168
Series 1	120	920	2330	3090	3660	3440	4120
2	150	1250	3060	3720	3730	4600	4500
3	110	1050	3160	3710	4130	4250	4400
4	120	950	2580	3350	4340	4200	5300
5	90	830	1840	2920	4050	3800	4200
Average	120	1000	2590	3360	3980	4060	4500

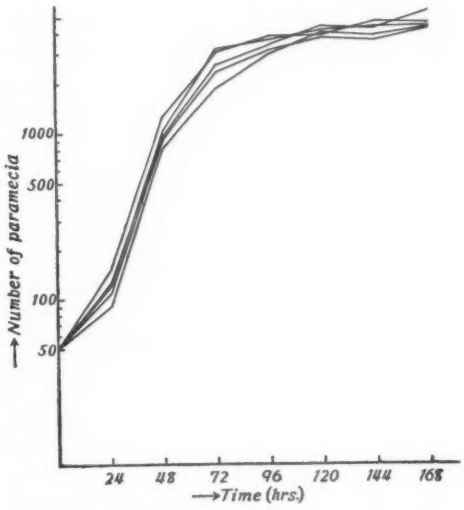


Fig. 1. Graphic presentation of Table I.

2) Average fission rate

To obtain a simple method for evaluating the effect of the material on the growth of *Paramecium*, a numerical presentation of the growth rate was introduced, that is, the author has defined the " average fission rate " by the equation

$$N_2=2^xN_1,$$

where, N_1 is the number of *Paramecia* before, and N_2 after, a given 24 hours' interval. Hence, the average fission rate x can be computed as follows.

$$x=\frac{1}{\log 2}(\log N_2-\log N_1)$$

Average fission rates (hereafter to be abbreviated often as AFR) in the standard growth calculated from the values in Table I are given in Table II. The AFR's were, on an average of five series of the experiment, 1.2, 3.1 and 1.4 in the first, the second and the third day respectively, and as small as 0.0 to 0.4 after the fourth day. The time trend of the AFR is shown in Fig. 2.

TABLE II

Average Fission Rate in the Standard Growth

Day	I	II	III	IV	V	VI	VII
Series 1	1.26	2.94	1.34	0.41	0.24	-0.09	0.26
2	1.58	3.06	1.29	0.28	0.00	0.30	-0.03
3	1.14	3.25	1.59	0.23	0.15	0.04	0.05
4	1.26	2.98	1.44	0.38	0.37	-0.05	0.34
5	0.85	3.21	1.15	0.67	0.47	-0.09	0.14
Average	1.2	3.1	1.4	0.4	0.3	0.0	0.2

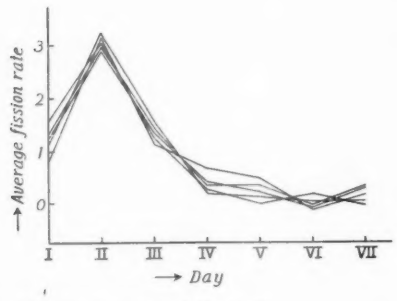


Fig. 2. Graphic presentation of Table II.

Method for Judging the Inhibitory Effect

The materials were tested for the effects on the growth of *Paramecium* in the second 24 hours' interval of the cultivation, where, as shown in the preceding chapter, the AFR of the organism was the highest.

Five test tubes were taken at random from 100 to 300 (the number to be varied in proportion to the number of the materials to be tested) test tubes, in each of which 50 *Paramecia* had been cultivated just for 24 hours, and the num-

ber of *Paramecia* in each of them was calculated in the manner as described before. The five numbers thus obtained were then averaged (N_1, the value must fall within the standard range of 120 ± 70[5]).

Next, the other five test tubes were taken, in each of which was added the given material. They were then shaken well and subjected to the further 24 hours' cultivation together with the other tubes. Exactly after 24 hours, the above-mentioned five test tubes were taken, and the numbers of *Paramecia* in them were calculated respectively, and averaged (N_2). Simultaneously the similar procedure was made on five of the remaining test tubes and the corresponding value N_1' was obtained for comparion.

From the values N_1, N_2 and N_2', the AFR's x and x', with and without the addition of the test material respectively, were calculated by the formula given in the preceding chapter. Then the judgement of the effects was carried out as follows: (1) x', the control AFR, must fall within the standard range of 3.1 ± 0.4[5]. When this was not realized, the data obtained in this experiment should be rejected and the experiment must be repeated, because some disturbances must have changed the given condition. (2) When the above condition had been satisfied, the grade of the inhibitory effect of the material was determined as - - — (no inhibition), when x was 2.7 or more; +, when x was 2.0~2.7 (2.0 or more, but less tan 2.7); ++, x being 1.0~2.0; +++, x being 0.0~1.0; ++++, when the number N_2 of *Paramecia* was smaller than the number N_1 and so x was calculated as a negative value, in consequence of the cytolysis of the dead organisms; ++++, when all of the organisms disappeared.

Materials and Results

Materials were prepared from the tissue of gastric carcinoma which had been obtained by operation. Each of the tissues used was later studied histologically, and the diagnosis ascertained.

From the unaffected mucous membrane of the stomach with peptic ulcer, which will be referred to hereafter as normal gastric mucosa, the corresponding materials were also prepared for comparison.

1) Aqueous extract of the tissue

a) *Fresh extract.* Eight cases of the gastric carcinoma tissue and eight of the normal gastric mucosa were extracted respectively with distilled water as follows.

A portion of the tissue was dissected from the resected stomach immediately after operation, washed shortly with water to remove the slime, blood and necrotic matter, wiped with gauze, and weighed. The tissue was then cut into small pieces and homogenized by the aid of an electric homogenizer. To the homogenate was added twice as much (in weight) distilled water and the mixture was subjected to a shaking extraction for 48 hours, and centrifuged.

The extract, i.e. the clear supernatant here obtained, was tested for the effects on the growth of *Paramecium*.

Concentration of each extract was estimated, on the other hand, by drying exactly 1 cc. of the extract.

TABLE III

Test with the Fresh Aqueous Estract

Case	No.	Concentration of the extract (mg./cc)	Results* with 3.0 cc.	1.0 cc.	2.5 cc.	Control N_1	AFR
Gastric carcinoma	1	31	###	###	+ (2.1)	120	3.1
	2	21	###	## (1.4)	+ (2.8)	130	3.3
	3	34	###	###	+ (2.4)	140	3.1
	4	37	###	## (1.0)	## (1.8)	110	3.2
	5	45	###	###	## (1.7)	120	2.8
	6	32	###	###	+ (2.2)	120	2.8
	7	28	###	###	− (2.7)	110	3.1
	8	28	## (0.7)	− (3.0)	− (3.0)	120	3.4
Normal gastric mucosa	1	31	###	+ (2.4)	− (2.8)	120	3.1
	2	53	###	## (1.5)	− (3.3)	150	3.1
	3	33	###	+ (2.0)	− (3.0)	150	3.1
	4	42	###	− (2.9)	− (3.3)	150	3.1
	5	28	## (0.8)	− (3.1)	− (3.4)	150	3.1
	6	44	###	## (1.2)	+ (2.1)	120	2.8
	7	20	+ (2.5)	− (3.4)	− (3.2)	110	3.1
	8	38	## (1.7)	− (2.8)	− (3.1)	120	3.4

* Explanation of signs is given in the text. Figures in parentheses show the average fission rates.

Results are shown in Table III. The growth of *Paramecium* was inhibited when 2 cc. of the extract were added to the culture medium. Though this effect was observed in all the cases tested, there were seen some differences in intensity of the inhibition between the cancer cases and the normal mucosa cases. In the cases of carcinoma, seven out of eight cases caused the complete disappearance or decrease in number of the organisms (inhibition of grade ### or ###, respectively) and the remaining one the inhibition of grade ##, while in the normal mucosa cases, those which showed the inhibition of over ### were five out of eight cases, and the effects of the other three were ##, ## and +, respectively.

Inhibition of the growth by the addition of 1 cc. of the extract was observed in seven out of eight cancer cases and in four of the eight normal mucosa cases, the intensity of which was over ## in the former and under ## in the latter.

With 0.5 cc. of the extract, five out of the eight cancer cases showed the inhibition ranging +~##, while none of the control cases were effective besides the one which showed the slight inhibition of grade +.

As stated above, the aqueous extract of the gastric carcinoma tissue inhibited the growth of *Paramecium* more markedly than that of the normal gastric

mucosa at various doses. Here, however, attention should be given to the fact that the concentration of the solid matter in the extract is, as seen in Table III, very variable. Therefore, a relation of the inhibitory effect of the extract to its final concentration in the culture medium was studied, with the results shown in Table IV. Being compared at the same concentration, the extracts prepared from the tissue of gastric carcinoma were obviously more intense in inhibition than those of the normal gastric mucosae.

TABLE IV

Relation of the Inhibitory Effect of the Fresh Aqueous Extract to their Concentraion

	No. of cases							
Concentration* (1:)→	100～250		250～500		500～1000		1000～	
Grade of inhibition ↓	Carci-noma	Normal mucosa	Carci-noma	Normal mucosa	Carci-noma	Normal mucosa	Carci-noma	Normal mucosa
###	6	1	1					
###	1	4	4					
##	1	1						
##		2	2	1	2			
+				4	3			
−			1	4	2	6	1	1
Total	8	8	8	9	7	6	1	1

* Final concentration of the solid matter of the extract in the culture medium.

b) *Dried powder of the extract.* Exactly speaking, the effects of the materials on the growth of *Paramecium* can be compared with each other only in the experiment, which consists of measuring the fission rates of the organism in the culture media, each of which contains one of the materials accurately at the same concentration. This procedure will be possible if the dried matter of the extract is available for the experiment. Thus a preliminary test was carried out to see whether the effective substances in the aqueous extract of the tissue suffer no change in effects when dried to powder.

The remaining portions of the aqueous extracts (three of the gastric carcinoma cases and three of the normal gastric mucosa cases were chosen) were condensed separately under reduced pressure and at the temperature below 40°C., dried to powder in a vacuum desiccator, and weighed. To the powder thus prepared was added a proper amount of distilled water to make a solution having exactly the same concentration as that of the original aqueous extract, and the solution was tested for the effect to be compared with that of the fresh aqueous extract.

Results are shown in Fig. 3. In all the cases tested, the curves of the AFR with two sorts of the material were almost similar to each other, suggesting

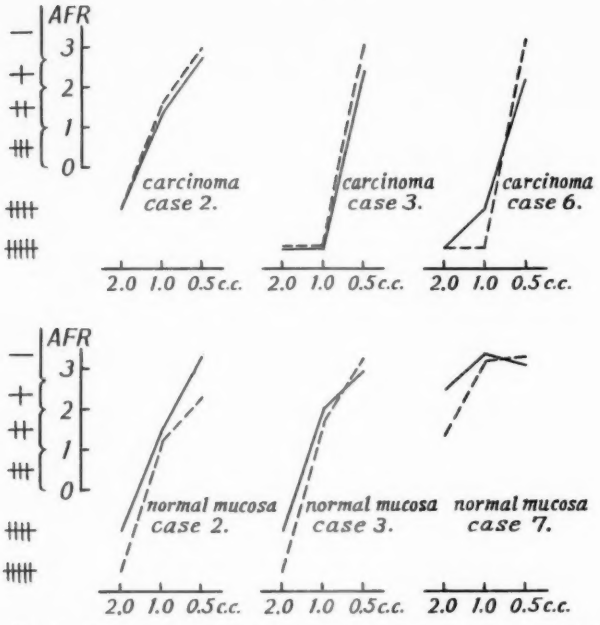


Fig. 3. Test with two sorts of the material: fresh aqueous extract (solid line) and the redissolved solution of dried extract (broken line).

that the procedure of condensing and drying does not affect the effective substances in the aqueous extract of the tissue.

2) Dried aqueous extract of the tissue

In view of tle fact clarified above, further experiments were carried on with the dried matter of the aqueous extract of the tissue (hereafter to be referred to simply as the extract). Moreover, the dried powder of the tissue prepared by grounding the dried fine slices of the tissue was used as the extraction material, for the convenience of preservation.

First, the extracts were prepared from 19 cases of the gastric carcinoma tissue and from 12 cases of the normal gastric mucosa as follows. Each dried powder of the tissue was extracted with 10 times as much distilled water with constant shaking for 48 hours; the extract was separated by centrifugation, condensed under reduced pressure at the temperature lower than 40°C,. and dried to powder in a desiccator (cf. Table V).

Fourty, 20 or 10 mg. of the extract thus obtained were added to the culture medium (10 cc. in volume) to make a solution of 1 :250, 500 or 1000 respectively and the growth of *Paramecium* was tested in them.

Results are summarized in Table VI. At 1 :250, all the extracts of the cancer cases presented the marked inhibition, 16 cases (84.2%) of which caused the disappearance or a decrease in number of the organisms (inhibition ### or

TABLE V

Test with the Dried Aqueous Extract—Materials

Case		Nature of the tumor			Amount of	
		Size ($cm.^2$)	Gross appearance	Microscopic diagnosis	the tissue used (g.)	the extract obtained (mg.)
Gastric carcinoma	1	6.5× 5.0	type II	Car. solid.	7 (1.7)*	410
	2	6.0× 5.0	III	solid.	7 (1.5)	320
	3	6.0× 5.0	II	aden.	14 (3.1)	770
	4	7.0× 7.0	II	aden.	27 (5.2)	1570
	5	5.0× 3.5	III	solid.	24 (4.6)	940
	6	5.0× 7.0	II	solid.	12 (1.5)	680
	7	14.0×12.0	IV	aden.	88 (16.7)	4900
	8	5.0× 5.0	II	aden.	6 (1.1)	330
	9	3.5× 3.0	II	aden.	8 (1.1)	610
	10	12.0× 8.0	II	aden.	20 (7.6)	780
	11	4.0× 4.5	II	aden.	15 (3.3)	760
	12	10.0× 7.5	II	solid.	16 (3.2)	790
	13	7.0× 8.5	II	solid.	30 (6.0)	1660
	14	9.0× 8.0	II	solid.	20 (4.3)	970
	15	4.0× 3.5	II	solid.	5 (1.2)	400
	16	3.0× 6.0	II	solid.	11 (2.0)	460
	17	4.0× 7.0	II	aden.	10 (2.3)	860
	18	3.0× 4.0	II	aden.	10 (2.2)	650
	19	5.0× 3.5	III	aden.	15 (3.6)	810
Normal gastric mucosa	1				15 (4.3)	490
	2				15 (4.7)	1640
	3				10 (2.7)	700
	4				50 (8.0)	1530
	5				40 (7.5)	1250
	6				36 (9.2)	540
	7				42 (8.7)	930
	8				18 (3.8)	460
	9				15 (3.1)	850
	10				15 (2.1)	440
	11				15 (3.8)	1200
	12				13 (3.1)	860

* Wet weight with dry weight in parenthesis.

###, respectively), while only four (33.3%) of the 12 control cases showed the inhibition over ###.

At 1:500, inhibition was observed in 17 (89.5%) out of the 19 cancer cases and in nine (26.3%) of the 12 normal mucosa cases, and the intensity

TABLE VI

Test with the Dried Aqueous Extract—Results

	Case	Results* at			Control	
		1:250	1:500	1:1000	N_1	AFR
Gastric carcinoma	1	###	###	+ (2.6)		
	2	###	## (0.7)	− (3.2)		
	3	###	+ (2.1)	− (2.9)		
	4	###	## (1.4)	− (2.8)		
	5	###	## (0.9)	− (3.0)		
	6	###	## (1.3)	− (3.1)		
	7	###	## (1.0)	## (1.7)		
	8	## (0.5)	## (1.9)	− (2.9)		
	9	###	###	## (1.9)		
	10	###	− (3.0)	− (3.2)		
	11	## (0.0)	## (1.1)	− (3.2)		
	12	###	###	− (2.8)	110	3.2
	13	###	## (0.6)	− (2.8)		
	14	###	## (0.5)	## (0.9)		
	15	## (1.3)	− (3.2)	− (3.0)		
	16	###	+ (2.2)	− (3.5)		
	17	###	###	+ (2.1)		
	18	###	## (0.8)	+ (2.6)		
	19	###	###	## (1.0)		
Normal gastric mucosa	1	###	## (0.7)	+ (2.6)		
	2	## (1.4)	+ (2.2)	− (3.2)		
	3	## (0.1)	+ (2.1)	− (3.2)		
	4	+ (2.4)	## (1.7)	− (3.3)		
	5	+ (2.6)	## (1.3)	− (2.8)		
	6	## (0.7)	− (3.1)	− (3.0)		
	7	## (1.3)	− (2.9)	− (3.5)		
	8	+ (2.3)	+ (2.5)	− (3.0)		
	9	## (1.8)	− (3.0)	− (3.3)		
	10	###	+ (2.5)	− (3.2)		
	11	###	+ (2.6)	− (3.0)		
	12	###	###	+ (2.6)		

* Figures in parentheses show the AFR.

of the effect was much larger in the former than in the latter.

Even at 1:1000, seven (36.8%) out of the 19 cancer cases showed the perceptible inhibition, while the effect was seen in only two (17.7%) of the 12 normal mucosa cases.

From the results obtained hitherto, it may be said, on the dried powder

of the extract as well as on the fresh extract, that the aqueous extract of the gastric carcinoma tissue is more abundant in substances inhibiting the growth of *Paramecium* as compared with that of the normal gastric mucosa.

Then, the data obtained from the carcinoma extracts were rearranged according to the size, the form or the histological feature of the tumor from which the material had been extracted, and the relation of the inhibitory effect of the extract to these natures of the tumor was studied. But, as shown in Table VII, no significant difference was seen in the inhibitory effect between 11 cases of larger tumor (the size being over 25 cm^2. in square measure) and eight of the smaller tumor (the size being under 25 cm^2.), between 15 cases of the tumor of Borrmann's types I or II and four of the types III or IV, and between 10 cases of carcinoma adenomatosum and nine of carcinoma solidum.

TABLE VII

Relation of the Inhibitory Effect of the Extract to the Nature of Tumor

	No. of cases					
Nature of the tumor→	Size		Gross appearance		Micro. diagnosis	
Grade of inhibition* ↓	small	large	I or III	III or IV	C. aden.	C. solid.
###	2	3	4	1	3	2
###						
##	2	3	4	2	2	4
++	1	4	3	1	3	1
+	1	1	2		1	1
−	1	1	2		1	1
Total	7	12	15	4	10	9

* At 1:500.

3) Considerations on the effective substances

Aqueous extract of the tissue was subjected to various chemical and physical treatments and the changes in intensity of the inhibitory effect were followed up.

a) *Mixture of the extract.* As the experiments were carried out hereafter with the mixture of the dried powders of the extracts, preliminary tests were run as follows. Dried aqueous extracts of 19 cancer cases and those of 12 normal mucosa cases were combined (the mixture will be referred to simply as the carcinoma extract or the normal mucosa extract). Results of the inhibition with these materials are shown in Fig. 4. Grade of the inhibition with the carcinoma extract was ### at 1:250, ++ at 1:500, and negative first at 1:1000, whilst the normal mucosa extract presented the inhibition only at 1:250.

Thus, differences in the inhibitory effect between two materials: cancer material and the normal mucosa material, were observed also with the mixture of the dried aqueous extracts.

b) *Dialysis.* Eighteen and three-tenths g. of the carcinoma extract were dissolved in 10 times as much distilled water and dialyzed in a cellophane bag against distilled water for 48 hours, during which time the dialyzate was replaced with distilled water every six hours.

The dialyzates here obtained were combined together, condensed under reduced pressure and dried (yield 6520 mg.). The dialyzed liquid, on the other hand, was centrifuged, a portion of the supernatant (1/10 out of 306 cc.) was condensed and dried (yield 320 mg.), while the centrifugate was totally dried to powder (yield 1750 mg.).

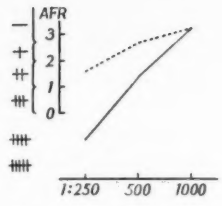


Fig. 4. Test with the mixture of the aqueous extracts. Solid line, carcinoma extract; dotted line, normal mucosa extract.

Corresponding materials were prepared from 5.5 g. of the normal mucosa extract in a quite similar manner. Yields of the dialyzate, the dialyzed substance (1/5 volume of the dialyzed liquid was used here) and the precipitate after dialysis were 3020 mg., 210 mg. and 430 mg., respectively.

Results with these fractions are shown in Fig. 5. The dialyzed matter of the carcinoma extract presented the inhibition of the growth at the dilutions within 1:1000, intensity of which ranged from + to ###, while that of the normal mucosa extract was effective only at as low a dilution as 1:250. On the other hand, the dialyzate of the extract was more intense in inhibition than the dialyzed matter, being effective at dilutions up to 1:2000 in the cancer material and to 1:500 in the normal mucosa material. Precipitate after dialysis was less effective, inhibition being observed at dilutions within 1:500 in both the materials.

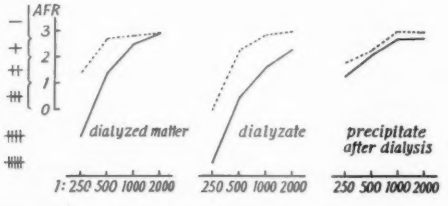


Fig. 5. Test with the three fractions of the aqueous extract. Solid line, carcinoma material; dotted line, normal mucosa material.

From these results it may be concluded that the dialyzate as well as the dialyzed matter of the carcinoma extract should contain the substances inhibiting the growth of *Paramecium*, in other words, there should exist at least two sorts of the effective substance in the aqueous extract of the gastric carcinoma tissue: dialyzable and non-dialyzable ones.

c) *Fractionation of the dialyzed matter.* The dialyzed matter of the carcinoma extract was fractionated in the manner, by which Inoue[6] of our clinic had pre-

pared the substance responsible for the skin reaction of cancer. The procedure may be outlined as follows. To 378 cc. of the dialyzed liquid (being equivalent to about 16.5 g. of the dried aqueous extract) were added drops of diluted hydrochloric acid to make the pH of the solution 4.2. After being left to stand for an hour, the solution was centrifuged, and the centrifugate was dried to powder after being washed thrice with distilled water whose pH had been adjusted to 4.2 with HCl ($Ppt_{pH\ 4.2}$, 435 mg.). The supernatant at the centrifugation and the washings of the centrifugate were combined together, a 1/5 portion of which was dried after being condensed under reduced pressure ($Sup_{pH4.2}$, 435 mg.).

The remaining large portion of the liquid above-mentioned was then acidified further to pH 2.0 with HCl, and the precipitate was separated by centrifugation after an hour, washed thrice with distilled water containing HCl at pH. 2.0 and dried ($Ppt_{pH\ 2.0}$, 8 mg.). The supernatant, 377 cc. in volume when combined with the washings of $Ppt_{pH\ 2.0}$, was neutralized and then saturated fully with ammonium sulfate (285 g. were required at 20°C.) and centrifuged.

The the centrifugate was washed twice with a saturated solution of $(MH_4)_2SO_4$, mixed with a small amount of distilled water and dialyzed against running water, while the supernatant at the centrifugation and the washings of the centrifugate were combined together and dialyzed also in a similar manner. After the dialysis had been completed, i.e. when Nessler's reaction for NH_4^+ gave no more positive result with the dialyzed liquid (about 48 hours were needed), the two liquids were condensed respectively under reduced pressure and dried to powder ($Ppt_{(NH_4)_2SO_4}$, 435 mg.; and $Sup_{(NH_4)_2SO_4}$, 350 mg.).

Results obtained with these fractions except $Ppt_{pH\ 2.0}$, which was too small in yield to be tested here, are shown in Fig. 6. $Sup_{(NH_4)_2SO_4}$ was the strongest in effect, its intensity being ### at 1:250 and ++ even at 1:2000, whilst $Ppt_{(NH_4)_2SO_4}$ was far less effective. $Sup_{pH\ 4.2}$, the mother fraction of the $Ppt_{pH\ 2.0}$, $Ppt_{(NH_4)_2SO_4}$ and $Sup_{(NH_4)_2SO_4}$, was also effective up to 1:2000, while $Ppt_{pH\ 4.2}$ presented only a slight inhibition within 1:500.

From these results it may be concluded that the effective substance in the dialyzed matter of the carcinoma extract should have been transferred, precipitated neither by acidification nor by full saturation with ammonium sulfate, into $Sup_{(NH_4)_2SO_4}$ through the fraction $Sup_{pH\ 4.2}$.

d) *Test with the dialyzate.* i) Solubility in organic solvents. One hundred and sixty mg. portion of the dialyzate of the carcinoma extract was put in 20 cc. of an organic solvent (benzene, chloroform, acetone, ether, amyl alcohol and ethyl alcohol were used here), agitated well and centrifuged after being left to stand for an hour at room temperature. The centrifugate was washed with the solvent used and then dried (insoluble part). The supernatant at the centrifugation and the washings of the centrifugate were combined and dried after being condensed under reduced pressure (soluble part).

When any of the substances thus prepared is dissolved into 4 cc. of distilled water, the solution may contain the substance equivalent to 160 mg. of the

mother fraction (the dialyzate of the carcinoma extract), in other words, the solution corresponds to a 1:25 solution of the dialyzate of the carcinoma extract. Such a solution was prepared from each of the materials, diluted to various degrees and tested. The results are summarized in Fig. 7.

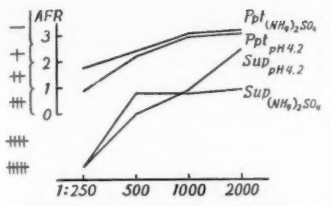


Fig. 6. Test with the fractions of the dialyzed carcinoma extract.

ii) Treatments with ion-exchange resins. Thirty g. of Amberlite IR 120, a cation exchange resin, were washed first with 50 cc. of 2 *N* HCl and then several times with deinonized water, put into 50 cc. of deionized water containing 1 g. of the dialyzate of the carcinoma extract and agitated. Then the liquid was decanted and the resin was washed thrice, each time with 50 cc. of deionized water. The decantate and the washings of the resin were combined, condensed under reduced pressure and dried (Amb. IR 120-unadsorbed fraction, 170 mg.).

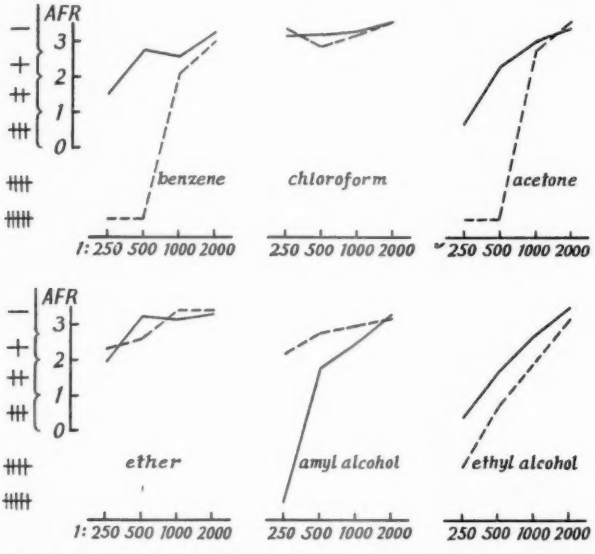


Fig. 7. Test with the dialyzate of the carcinoma extract after treatment with organic solvents. Solid line, soluble part ; broken line, insoluble part.

One g. of the dialyzate of the carcinoma extract was also treated with Amberlite IR A-410, an anion exchanger, which had been washed beforehand with 2 *N* NaOH and with deionized water, in such a manner as in the case with the cation exchanger. Yield of the Amb. IR A-410-unadsorbed fraction was 180 mg.

As shown in Fig. 8, the Amb. IR 120-unadsorbed fraction was found to be markedly less effective as compared with the original dialyzate of the carcinoma extract, while the Amb. IR A-410-unadsorbed fraction was as much effective as the mother substance (cf. Fig. 5), suggesting that the substances, which exist in the dialyzate of the carcinoma extract and are responsible for the inhibition of the growth of *Paramecium*, are well adsorbed by a cation exchanger but not by an anion exchanger.

Fig. 8

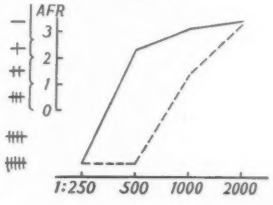


Fig. 9

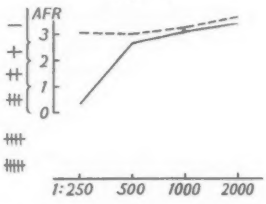


Fig. 8. Test with the dialyzate of the carcinoma extract after treatment with iron-exchange resins. Solid line, Amb. IR 120-unadsorbed fraction; broken line, Amb. IR A-410-unadsorbed fraction.

Fig. 9. Test with the dialyzate of the carcinoma extract after heating (solid line) or incineration (broken line).

iii) Effect of heating or of incineration. One hundred and sixty mg. of the dialyzate of the carcinoma extract were dissolved in 4 cc. of distilled water to make a 1:25 solution, boiled under refleux for 30 minuts and then allowed to cool.

On the other hand, another 160 mg. portion of the substance was incinerated thoroughly in a porcelain crucible and dissolved also in 4 cc. of distilled water to make the solution equivalent to a 1:25 solution of the dialyzate of the carcinoma extract.

Results with these two solutions are presented in Fig. 9. It was revealed that the inhibitory effect of the dialyzate is decreased by 30 minutes' heating at 100°C., and lost almost wholly by incineration.

Discussion

As reported previously, the extract of the gastric carcinoma tissue inhibits the movement of *Paramecium*, and a basic substance of low molecular weight and an unsaturated fatty acid are responsible for this effect[1)2)].

In relation to the fact given above, the present work was undertaken to study whether the tissue of carcinoma contains also substances inhibiting the growth of *Paramecium*.

Paramecium is a species of Protozoa belonging to the *Ciliata*, its reproduction being, within a single strain, carried by asexual division[7)]. Though various methods have been presented for observing the reproduction of

this organism, the test tube culture in the closed system was adopted here, which consists of cultivatig a given number of the organism with a given amount of medium in a test tube under a fixed condition, and of observing the changes in its number from time to time. The culture medium used is a baked lettuce infusion inoculated with *Aerobacter aerogenes*, the same as was used by Sonneborn[8] and Koizumi *et al.*[9]

First, the standard curve of the growth was established (Fig. 1). The number of *Paramecia* increased slowly in the first day of the culture, much rapidly in the second day and then the rate of the growth became smaller and smaller, reaching the equilibrium on the fifth day.

For detecting the effects of the material on the growth of *Paramecium*, it may be theoretically better to cultivate the organism with the medium containing the material to be tested, and to compare the curve of the growth obtained there with the standard one.

However, this method can not be applied in the present work because the materials to be tested are rich in organic substances which are too ready to be denatured or putrefied. This was the reason why the author had planed an unusual, or simplified, method.

Thus, in view of the fact that the growth of *Paramecium* is most remarkable in the second day of the culture, the material was added in the culture medium 24 hours after the commencement of the culture and the rate of the growth within the next 24 hours' interval was measured, and this was compared with the standard. Moreover, a numerical presentation of the rate of the growth, i.e. a conception of the average fission rate, was introduced from a theoretical point of view, which enabled one to grade the intensity of the inhibitory effect into five classes : $+ \sim \#$.

Tests were carried out first with the aqueous extract of the fresh tissue in which the water-soluble substances of the tissue must have been transferred almost unchanged in nature. Results obtained here revealed that the extracts of the gastric carcinoma tissues are obviously more intense in inhibition than those of the normal gastric mucosae, that is to say, that the tissue of gastric carcinoma must contain the substances inhibiting the growth of *Paramecium* more abundantly as compared with the normal gastric mucosa.

Needless to say, it may be a great convenience, if possible, to carry out experiments with the dried material of the extract, not with the extracted liquid itself, for the exact measurement and the long preservation of the material are both easy in this case.

After preliminary confirmation of the fact that the substances responsible for the inhibition of the growth of *Paramecium* are not affected by drying, the dried aqueous extracts were prepared from 19 cases of the gastric carcinoma tissue and from 12 cases of the normal gastric mucosa. And, as

might have been expected, the results obtained with them coincided with those of the fresh aqueous extract of the tissue.

Intensity of the inhibitory effects of the extract was proved to not depend upon such a nature of the carcinoma as the size, the gross appearance or the microscopic feature of the tumor. This may be an interesting fact suggesting that the inhibitory substance should be contained generally in the carcinoma tissue, irrespective of such a nature of the tumor.

Then the substance responsible for the effect was considered by fractionating the carcinoma extract finely. As the inhibitory effect was recognized both in the dialyszate and in the dialyzed matter of the extract,it is obvious that there should exist at least two sorts of the effective substance. Further studies have revealed that the effective substance in the dialyzed matter is a substance which can be precipitated from the solution neither by acidification nor by salting out with ammonium sulfate, and presumably a carbohydrate fraction ; while the substance in the dialyzate is a basic substance of low molecular weight.

In the present series, Oh-Uti *et al.*[9] have previously demonstrated that a carbohydrate fraction, which was prepared from the tissue of gastric carcinoma in a quite similar manner as used in the present work, induces anemia in rabbits when injected into the vein. According to Inoue[6], the same substance is also responsible for the skin reaction of cancer with the carcinoma extract. It is an interesting fact that the same substance contained in the tissue of carcinoma presents various biological activities obviously differing from each other in mechanisms of the action.

As concerns the basic substance of low molecular weight in the carcinoma tissue, Maki[10] of our clinic has found that it inhibits the beats of the excised frog heart. Following this investigation, inhibition of the ciliary movement of the oyster gill and of the movement of *Paramecium* have been recognized in similar substances, by I. Yamaguchi and Oh-Uti[11] and by Oh-Uti *et al.*[1] respectively. The substance obtained by the author was quite similar to those described above, for example in the solubility to organic solvents, in the mode of adsorption to the ion-exchangers or in the heat lability, and may be identical with them.

In conclusion some considerations will be made on the relation of the two activities on *Paramecium*, inhibition of the growth and that of the movement.

The carbohydrate fraction, one of the substances inhibiting the growth of *Paramecium*, did not immobilize this organism[3], therefore this fraction is considered to affect the growth of *Paramecium* primarily, not in consequence of an inhibition of the movement. On the other hand, the basic substance of low molecular weight inhibited both the growth and the movement. Here, however, the highest possible dilution to realize inhibition of the

growth was much higher than that required to the immobilization, indicating that the substance may possess the activity to inhibit the growth as well as the movement of *Paramecium*.

Summary

1. Aqueous extracts of the gastric carcinoma tissues inhibited the growth of *Paramecium* more intensely than those of the normal gastric mucosae.

2. A carbohydrate fraction and a basic substance of low molecular weight were responsible for this effect.

3. Intensity of the inhibitory effect of the cancerous extract was not dependent upon the nature of the tumor from which the extract had been prepared.

References and Notes

1) Oh-Uti, Hasegawa, Yamaguchi, I. & Abe, Tohoku J. Exper. Med., 1957, **67**, 87.
2) Yamaguchi, I., Kamma & Oh-Uti, *ibid.*, 1959, **69**, 185.
3) Oh-Uti, Abe & Yamaguchi, I., *ibid.*, 1957, **67**, 91.
4) Koizumi & Hiraga, J. Ecol. Soc. Japan (Jap.), 1956, **6**, 16.
5) Rejection limits at 5 per cent level of significance were used.
6) Inoue, to be published.
7) Wichterman, The Biology of paramecium, New York, 1953, p. 253.
8) Sonneborn, J. Exp. Zool., 1950, **113**, 87.
9) Oh-Uti, Suzuki & Yamaguchi, I., Tohoku J. Exper. Med., 1958, **67**, 115.
10) Maki, *ibid.*, 1956, **63**, 121.
11) Yamaguchi, I. & Oh-Uti, *ibid.*, 1957, **67**, 73.

The Tohoku Journal of Experimental Medicine, Vol. 69, No. 4, 1959

Tuberculin-Polysaccharide in the Blood of Tuberculous Infants and Children; Influence of Chemotherapy upon it.

(Studies on Tuberculosis in Infancy and Childhood. 6th Report)

By

Tsuneo Arakawa, Mizuo Oota and **Yuji Sato**
(荒川 雅男) (太田 瑞夫) (佐藤 雄治)

From the Department of Pediatrics, Faculty of Medicine, Hirosaki University, Hirosaki; Director: Prof. Ts. Arakawa

(Received for publication, January 31, 1959)

It was reported by us that the identification of the erythrocytes sensitized with the tuberculin-polysaccharide in circulating blood[1)2)3)] or the approximate estimation of the tuberculin-polysaccharide in serum[4)] was of value for evaluation of *activity* of the tuberculous foci in infancy and childhood.

In the present paper we shall deal with the results of tuberculous infants and children subjected to chemotherapy in whom the sensitized erythrocytes and/or the tuberculin-polysaccharide in serum had been identified.

Experimental

Material and method

The materials were 49 patients under the age of 11 years, and they were divided into 3 groups (Groups I, II and III). Group I included 25 cases in which the erythrocytes sensitized with the tuberculin-polysaccharide in circulating blood and/or the tuberculin-polysaccharide in the serum had been identified before chemotherapy was given. Group II included 16 cases in which the sensitized erythrocytes were not identified in circulating blood, but whose tuberculin-polysaccharide was identified in the serum prior to antituberculous treatment. Group III included 8 cases, in which, after identification of the sensitized erythrocytes and/or the tuberculin-polysaccharide in serum, specific antituberculous treatment was not tried at all, while blood examination was performed as in the cases of Groups I and II.

The identification of the sensitized erythrocytes in circulating blood

and the approximate estimation of the tuberculin-polysaccharide were carried out in the same way as reported in the preceding papers[3)4)]. The results of the estimation of tuberculin-polysaccharide in serum were obtained only in and after October 1958, because the actual method had not been accomplished before that time. The mark – in Tables I, II, and III means the absence of the tuberculin-polysaccharide in the serum, and the marks + and ++ in the same Tables mean approximately 118 γ% and 157 γ% of the tuberculin-polysaccharide respectively.[4)]

Types I, II and III of behavior of tuberculin-polysaccharide in the blood are of the following significance.[5)] Type I: The erythrocytes were being sensitized with tuberculin-polysaccharide and/or the tuberculin-polysaccharide was identified in the serum, while the antibody against the tuberculin-polysaccharide in the serum was not found by means of the Middlebrook-Dubos reaction. Type II: Tuberculin-polysaccharide was identified only in the serum, and the antibody was found by the Middlebrook-Dubos reaction. And Type III: Tuberculin-polysaccharide was not identified in the blood, while the antibody was found in the serum.

In all the cases subjected to blood examination, the Mantoux test was carried out by intracutaneous injection of 0.1 cc. of the old tuberculin of 1:2000.

The chemotherapy in the cases of Groups I and II was carried out as follows: Streptomycin (SM), 0.3–0.5 g. per day, was given intramuscularly every day or twice a week, para-aminosalicylic acid (PAS), 0.3–0.5 g/kg/day, and isoniazid (INH), 5–10 mg/kg/day were given orally every day. In the majority of the cases two of these three remedies were used combined. In a few cases, sodium-PAS was given intravenously by means of drop infusion of 300 cc. of 5% glucose solution in which 2–6 g. of it was dissolved just prior to the infusion.

Results and Discussion

Group I (Cf. Tables I and IV)

The effect of chemotherapy was seen most impressively in Cases 1, 12 and 21, in which, the erythrocytes having become free from the tuberculin-polysaccharide for the first time, the tuberculin-polysaccharide came then to be identified only in the serum; at last it even disappeared from the serum. Thus a complete disappearance of tuberculin-polysaccharide from the erythrocytes at first, and that from serum itself in the last stage,—that is to say—, the changes—from Type I through Type II to Type III—of the behavior of the tuberculin-polysaccharide in the blood were observed in all these cases. And these changes were generally accompanied by a clinical improvement (Cf. Table I).

The direct change from Type I to Type III—apparently without

Abreviations used in Tables I, II and III.

Anor.	Anorexia
At.	Atelectasis
Bronc.	Bronchitis
B.	On the both sides
Bp.	Bronchopneumonia
C.	Cough
C. dist.	Cough, distressing
den. Sh.	Dense shadows
Diar.	Diarrhea
Dec.	Decrease in shadows
Dyst.	Dystrophy
ev. d.	Every day
Ery. sens.	Erythrocytes sensitized with tucerculin-polysaccharide
F.	Fever
hil.	Hilar region
I.S.C.	Immune Serum C
Irri.	Irritability
Inc.	Increase in shadows
intrav.	Intravenous
INH 5 mg	INH 5 mg/kg/day
l.L.	Lower lobe
L.	On the left side
M.	Measles
No	No abnormality in X-ray findings
No co.	No complaints
No im.	No improvement
R.	On the right side
P.	Pallor
PAS 0.3 g	PAS 0.3 g/kg/day
S. dist.	Sleep disturbed
sl. F.	Slight fever
Sw.	Sweating
tw.	Twice a week
Tbc.	Tuberculous patients
T-P.	Tuberculin-polysaccharide
u.L.	Upper lobe
6/X-20/XI	From 6/X to 20/XI
6/X-,	From 6/X on,

passing through the stage of Type II—occurred in 21 cases (Nos. 2–9, 11, 13–20 and 22–25), only because a practical method for identifying Type II had not been devised yet at that time, but it is highly probable that these cases came over to Type III through the stage of Type II.

In Case No. 10, the tuberculin-polysaccharide in the serum had not disappeared in spite of a more or less long continued chemotherapy during more than 7 months, and it is rather interesting to note that this infant was in a close contact with his mother who was suffering from open tuberculosis of the lungs.

In Group I, 11 cases (Nos. 1, 3, 6–7, 10–11, 13–15, and 22–23) were subjected to monthly blood examination, in all of which the disappearance of the erythrocytes sensitized with tuberculin-polysaccharide occurred within one month of the onset of chemotherapy (Cf. Tables I and IV).

Group II (Cf. Tables II and V)

Our monthly blood examination showed that in 7 cases (Nos. 26, 28–30, 34–35 and 37) of Group II, the change from Type II to Type III occurred within one month of the onset of chemotherapy.

Group III (Cf. Tables III and V)

In one case (No. 44) of Group III, the sensitized erythrocytes which had been identified at the first blood examination, disappeared in lapse

TABLE I

Group I

No. of cases	Date	Age, years and months	Complaints and remarks	X-ray findings	Mantoux test	BCG-vaccination	Familial contact with Tbc.	Ery. sens.	Antibody against T-P. in serum (Titer of dilution of I.S.C.) 10 20 40 80 160 320	T-P. in serum	Treatment
1	6/X 1958	0.5	P. and Sl. dist.	Inc. hil. R.	2×2	−	+	+	− − − − − −	++	6/X-22/XII, INH 5 mg ev. d. SM 0.3 g tw. 6/X-8/XII, ev. d. 9/XII-22/XII
	20/XI	0.6						−	− − − − − −	+	
	8/XII	0.7						−	− − − − − −	++	
	22/XII		No co.					−	− − − − − −	−	
2	25/III 1958	0.5	Diar.	No	3×3	−	−	+	− − − − − −		25/III-27/XII, INA 5 mg ev. d.
	29/VII	0.9	M.					−	+ + + − − −		
	27/XII	1.2	No co.					−	+ + + − − −	−	
3	13/IX 1957	0.4	Anor. and F.	At. R.	3×2	−	+	+	− − − − − −		13/IX 1957-4/III 1958, SM 0.5 g tw. INH 5 mg ev. d. During Oct. 1957, PAS-Na 2 g given intrav. 21 times.
	15/X	0.5						−	− − − − − −		
	28/I 1958	0.7	M.	No				−	− − − − − −		
	4/III	0.9	No. co.		4×4			−	− − − − − −		
	27/V	0.11						−	+ − − − − −		
	17/XI	1.5						−	+ + + + − −	−	
4	4/XII 1957	0.11	sl. F.	Inc. hil. R.	3×3	−	−	+	− − − − − −		1/III-30/IX, SM 0.3 g tw. INH 10 mg ev. d.
	28/I 1958	1.0	M.					−	+ − − − − −		
	4/III	1.2		den. Sh. l.L.R.				−	+ − − − − −		
	15/IV	1.3	Diar.					−	+ + + − − −		
	20/V	1.4	C. distr.	At. u. L. R.	4×4			−	+ + − − − −		
	13/X	1.9	No. co.					−	+ + + − − −	−	
	8/XII	1.11						−	+ + + − − −	−	
5	17/VI 1958	0.6	Diar. and P.	den. Sh. l.L.R.	3×3	−	−	+	− − − − − −		17/VI-31/XII SM 0.3 g tw. PAS 0.3 g ev. d.
	27/XI	0.11						−	+ − − − − −	−	
	11/XII	1.0	Diar. and sl. F.					−	+ − − − − −	−	
	7/III 1957	0.5	Sl. dist. and sl. F.	Inc. hil. R.	15×21	−	+	+	− − − − − −		7/III-30/VIII 1957, INH 10 mg. ev. d. PAS 0.3 g ev. d.
	19/IV	0.6						−	− − − − − −		
	21/V	0.7						+	− − − − − −		

6	6/VIII	0.10	No co.	Dec.				−	−	−	−	−	−		
	24/IX	0.11						−	+	+	−	−	−		
	5/XI	1.1						−	−	−	−	−	−		
	23/XII	1.2	M.					−	+	−	−	−	−		
	28/I 1958	1.3						−	-	−	−	−	−		
	11/II	1.4						−	+	−	−	−	−		
	4/III	1.5	Auor.					−	+	−	−	−	−		
	8/IV	1.6						−	+	+	−	−	−		
	24/XI	2.0						−	+	+	+	+	−	−	
7	8/III 1957	0.9	P.	No	2×2	−	+	+	−	−	−	−	−		8/III-30/V 1957 INH 8 mg ev. d. 13/III-24/IV 1958, SM 0.25 g ev. d. 25/IV-30/×1958 SM 0.5 g tw. 12/III-30/X 1958 PAS 0.3 g ev. d.
	19/IV	0.10						−	−	−	−	−	−		
	12/III 1958	1.9	Bp.		0×0			−	+	−	−	−	−		
	9/V	1.11	sl. F.	No				−	−	−	−	−	−		
	3/VI	2.0	No co.					−	+	+	−	−	−		
	6/X	2.4						−	+	+	−	−	−	−	
	20/XI	2.5						−	+	+	+	−	−	−	
8	11/XII 1957	0.9	Dial.	No	11×13	−	−	+	−	−	−	−	−		7/II-1/III 1958, SM 0.5 g ev. d. and INH 10 mg ev. d. 2/III-18/IV 1958 SM 0.5 g tw. INH 10 mg ev. d.
	28/I 1958	0.10						+	−	−	−	−	−		
	21/II	0.11	Diar. and sl. F.					+	−	−	−	−	−		
	25/III	1.0						−	+	+	−	−	−		
	10/VI	1.3						−	+	−	−	−	−		
	2/X	1.7	Diar.					−	+	+	+	−	−	−	
9	18/III 1958	0.6	Anor.	No	15×14	−	+	+	−	−	−	−	−		20/V-12/XI SM 0.3 g tw. INH 5 mg ev. d.
	6/V	0.7						−	−	−	−	−	−		
	29/VII	0.9						−	−	−	−	−	−		
	12/XI	0.10	No. co.					−	−	−	−	−	−	−	
10	14/XI 1957	0.3	Sl. dist., Diar. and P.		0×0	−	−	+	−	−	−	−	−		1/III-30/X 1958 INH 5 mg ev. d. SM 0.5 g ev. d. 4/III-24/III, tw. 25/III-30/X
	10/XII	0.4						+	−	−	−	−	−		
	4/II 1958	0.6	M.					+	−	−	−	−	−		
	4/III	0.7						+	−	−	−	−	−		
	18/III							−	+	−	−	−	−		
	15/IV	0.8	No co.		4×6			−	+	−	−	−	−		
	13/V	0.9						−	+	−	−	−	−		
	10/VI	0.10						−	+	+	−	−	−		
	6/X	1.2	Diar. and C.					−	+	+	−	−	−	+	

Group I—*Continued*

No. of cases	Date	Age, years and months	Complaints and remarks	X-ray findings	Mantoux test	BCG-vaccination	Familial contact with Tbc.	Ery. sens.	Antibody against T-P. in serum (Titer of dilution of I.S.C.) 10 20 40 80 160 320	T-P. in serum	Treatment
11	18/II 1958	1.4	Anor. Diar. and sl. F.	Inc. hil. R.	2×2	−	+	+	− − − − − −		SM 0.3 g tw. 18/II-28/II, ev. d. 1/III-29/III, tw. 30/III-30/VII. 18/II-27/III INH 10 mg ev. d. 30/III-30/VII PAS 0.3 g ev. d.
	28/II							+	− − − − − −		
	11/III	1.5						+	− − − − − −		
	25/III							−	+ + + − − −		
	8/IV	1.6	No co.					−	+ + + − − −		
	6/V	1.7			6×6			−	+ + + − − −		
	30/X	2.0						−	+ + − − − −	−	
12	6/V 1958	1.4	F.	den. Sh. l.L.R.	2×2	−	+	+	− − − − − −		6/V-8/XII, SM 0.3 g tw. INH 5 mg ev. d. In June and July, PAS-Na, 2 g. intrav. 49 times.
	6/X	1.9						−	+ + + + − −	+	
	13/X							−	+ + + + − −	+	
	17/XI	1.10	No. co					−	+ + + + − −	−	
	8/XII	1.11						−	+ + + − − −	−	
13	22/II 1958	1.1	F. and Dyst.	Inc. hil. B.	0×0	−	−	+	− − − − − −		22/II-25/XII SM 0.5 g tw. PAS 0.3 g ev. d.
	11/III							−	+ + + − − −		
	15/IV	1.3	No co.					−	+ + + − − −		
	3/VI	1.5						−	+ + + − − −		
	30/X	1.9		Dec.				−	+ + + + − −	−	
	11/XII	1.11	Bronc.					−	+ + + + + +	−	
14	3/XII 1957	1.6	P. and sl. F.	No	9×10	−	+	+	− − − − − −		3/XII 1957-30/VI 1958 SM 0.5 g tw. INH 5 mg ev. d.
	7/I 1958	1.7						−	+ + − − − −		
	25/III	1.9						−	+ + − − − −		
	6/X	2.4	No co.					−	+ + + − − −	−	
	11/XII	2.6	sl. F.					−	+ + − − − −	+	
15	14/I 1958	1.2	sl. F.	Sh. l.L.R.	0×0	−	+	+	− − − − − −		14/I-2/X SM 0.3 g tw. PAS 0.3 g ev. d.
	18/II	1.3						−	− − − − − −		
	18/III	1.4	No co.		4×5			−	+ − − − − −		
	15/IV	1.5						−	+ − − − − −		
	10/VI	1.7						−	+ + − − − −		
	8/VII	1.8		No				−	+ + − − − −		
	20/X	1.10			3×3			−	+ + − − − −	−	

16	24/VI 1958	1.4	C.	Sh. l.L.R.	4×4	–	+	+	–	–	–	–	–		22/VII-22/XII SM 0.5 g tw. PAS 0.3 g ev. d.
	10/XI	1.9	No co.					–	–	–	–	–	–	–	
	12/XII	1.10						–	–	–	–	–	–	–	
17	28/I 1958	1.7	P. Diar. sl. dist.	No	3×3	–	–	+	–	–	–	–	–		28/I-30/IX SM 0.3 g tw. PAS 0.3 g ev. d.
	18/III	1.9						–	+	–	–	–	–		
	15/IV	1.10	M.					–	+	–	–	–	–		
	16/VI	2.0						–	–	–	–	–	–		
	6/X	2.4	No co.					–	+	+	+	–	–	–	
18	30/IV 1957	1.4	Anor. sl. F.	No	5×6	–	–	+	–	–	–	–	–		30/IV 1957-30/VI 1958 INH 5 mg ev. d.
	25/V	1.5						+	–	–	–	–	–		
	13/VIII	1.8						–	–	–	–	–	–		
	13/IX	1.9						–	–	–	–	–	–		
	15/X	1.10						–	+	+	–	–	–		
	12/XI	1.11						–	–	–	–	–	–		
	17/XII	2.0						–	+	+	–	–	–		
	4/II 1958	2.1						–	+	+	+	–	–		
	11/III	2.3	M.	No	2×2			–	+	+	+	–	–		
	25/III		sl. F.					–	+	+	–	–	–		
	20/X	2.10	No co.					–	+	+	+	+	+	–	
19	9/IV 1957	1.4	sl. F.	No	6×6	–	–	+	–	–	–	–	–		9/IV-30/VII 1957, INH 10mg ev. d. 25/IV-6/VIII 1958 PAS-Na, 2 g, intrav. 53 times. 21/VI-17/X 1958 SM 0.5 g tw.
	13/IX	1.10						–	+	–	–	–	–		
	4/XII	2.1						–	+	+	+	+	–	–	
	28/I 1958	2.2						–	+	+	+	+	–	–	
	25/II	2.3						–	+	+	+	+	–	–	
	15/IV	2.5						–	+	+	+	+	–	–	
	13/V	2.6						–	+	+	+	–	–	–	
	6/X	2.11	sl. F.					–	+	+	+	+	–	–	
20	25/VI 1957	2.2	Anor. sl. F.	No	3×3	–	+	–	–	–	–	–	–		25/VI-30/VIII 1957 PAS 0.3 g and INH 5 mg ev. d. 1/VIII-31/XIII 1958, SM 0.5 g tw. INH 10 mg ev. d. 1/X-11/XII 1958, PAS-Na, 2 g, intrav. 19 times.
	3/IX	2.5						–	+	+	–	–	–	–	
	1/X	2.6						–	+	+	–	–	–	–	
	6/V 1958	3.2	Anor. sl.	den. sh. l.L.R.	7×8			–	+	+	–	–	–	–	
	22/VII	3.4						–	+	+	–	–	–	–	
	10/XI	3.8	No co.					–	+	+	+	+	+	–	
21	21/V 1057	2.2	Anor. and sl. F.	No	26×29	–	+	+	–	–	–	–	–		21/V-3/IX 1957, INH 5 mg ev. d. 17/IX-, SM 0.5 g ev. d., PAS 0.3 g
	3/IX	2.6						–	+	+	–	–	–	–	

Group I—*Continued*

No. of cases	Date	Age, years and months	Complaints and remarks	X-ray findings	Mantoux test	BCG-vaccination	Familial contact with Tbc.	Ery. sens.	Antibody against T-P. in serum (Titer of dilution of I.S.C.) 10 20 40 80 160 320	T-P. in serum	Treatment
21	6/V 1958	3.2						−	+ + + + − −		ev. d.
	24/XI	3.8						−	+ + + + + +	++	
	11/XII	3.9	F.					−	+ + + + + −	−	
22	6/V 1958	3.9	F. Anor.	Inc. hil. B.	18×18	+	+	+	− − − − − −		10/V-30/X PAS 0.3 g, INH 5 mg ev. d., SM 0.5 g tw.
	24/VI	3.10						−	− − − − − −		
	4/XI	4.4	No. co.	Dec.				−	+ − − − − −	−	
23	5/XI 1957	5.3	Anor. sl. F.	Inc. hil. B.	34×28	+	+	+	− − − − − −		5/XI 1957- 30/VI 1958, SM 0.5 g tw., PAS 0.3 g and INH 5 mg ev. d.
	23/XII	5.4						−	+ + + + − −		
	4/III 1958	5.7						−	+ + + − − −		
	15/IV	5.8						−	+ + + − − −		
	13/V	5.9						−	+ + + − − −		
	4/IX	6.3	No co.					−	+ + + − − −	−	
24	19/VIII 1958	5.2	sl. F.	Inc. hil. B.	5×5	−	−	+	− − − − − −		19/VIII-22/XII, SM 0.5 g tw. PAS 0.3 g ev. d.
	6/X	5.4	No co.					−	+ + − − − −	−	
	24/XI	5.5						−	+ + − − − −	−	
	22/XII	5.6						−	+ + + + − −	−	
25	2/VIII 1957	6.3	Dyst. Ano. Diar.	No	0×0	−	+	+	− − − − − −		27/IX 1957-30/ VIII 1958 SM 0.5 g tw. PAS 0.3 g and INH 5 mg ev. d.
	22/X	7.5						−	− − − − − −		
	28/I 1958	7.8						−	− − − − − −		
	10/XI	8.6	No co.					−	− − − − − −	−	
	8/XII	8.7	Dyst.					−	− − − − − −	+	

of one month without any chemotherapy. On the other hand, in 6 cases of Group III, in which Type II had been established on the initial blood examination and monthly examination was carried out, the change from Type II to Type III within one month occurred in only 2 cases (Nos. 45 and 48), while in all the other 4 cases (Nos. 44, 46–47, and 49) Type II remained insistent and would not change to Type III for more than one month.

TABLE II

Group II

No. of case	Date	Age, years and months	Complaints and remaaks	X-ray findings	Mantoux test	BCG-vaccination	Familial contact with Tbc.	Ery. sens.	Antibody against T-P. in serum (Titer of dilution of I.S.C.) 10 20 40 80 160 320	T-P. in serum	Treatment
26	6/X 1958	0.8	Anor. Irri.	Inc. hil. B.	0×0	−	+	−	+ + + + + −	+	6/X-, SM 0.3 g tw. PAS 0.3 g ev. d..
	10/XI	0.9	No co.		4×3			−	+ + + + + +	−	
27	6/X 1958	0.11	C. distr.	No	2×2	−	−	−	+ + + + + −	++	6/X-1/XII, SM 0.5 g tw. PAS-Na, 2 g, intrav. 40 times
	1/XII	1.1	No. co.					−	+ + + + + −	−	
28	2/X 1958	1.3	Anor. Diar	Inc. hil. R.	22×16	−	−	−	+ + + + − −	+	2/X-13/XI, SM 0.3 g tw. PAS 0.3 g ev. d.
	13/XI	1.4	Anor.					−	+ + + + + +	−	
29	6/X 1958	1.3	Diar.	No	0×0	−	−	−	+ + + − − −	++	4/VIII-17/XI, SM 0.5 g tw. INH 5 mg ev. d. 2/XI-17/XI. PAS-Na, 2 g, intrav. ev. d.
	17/XI	1.4	No co.					−	+ + + + + −	−	
30	16/X 1958	3.5	F. C.	Sh. l.L.R.	0×0	−	−	−	+ + + + + −	+	16/X-, SM 0.3 g, INH 5 mg ev. d.
	19/XI	3.6	No co.					−	+ + + + + −	−	
31	12/VIII 1958	4.0	Anor. sl. F.	Inc. hil. B.	3×3	−	−	−	+ − − − − −		12/VII-11/XII, SM 0.5 g tw. PAS 0.3 g ev. d. 27/VIII-2/X, PAS-Na, 2 g, intrav. ev. d.
	2/X	4.3	No co.					−	+ + − − − −	−	
	24/XI	4.4	Bronc.					−	+ + − − − −	++	
	11/XII	4.5						−	+ + − − − −	−	
32	20/X 1958	4.0	Anor.	No	0×0	−	−	−	+ + + + + +	+	30/X, SM 0.5 g tw. PAS 0.3 g ev. d.
	8/XII	4.2	Anor.					−	+ + + + + −	−	
33	16/X 1958	5.0	Sw.	Inc. hil. B.	12×14	−	+	−	+ + + + + −	++	30/X-, SM 0.5 g tw. PAS 0.3 g ev. d.
	8/XII	5.2	No co.					−	+ + + + + −	−	
34	24/VIII 1958	6.0	sl. F.	No	6×6	+	+	−	+ + − − − −		Since about one year ago, SM 0.5 g tw. PAS 0.3 g ev. d.
	6/X	6.2						−	+ + − − − −	+	
	4/XI	6.3	sl. F.					−	+ + − − − −	−	
35	2/X 1958	6.0	Anor. sl. F.	Sh. l.L.R.	10×10	+	+	−	+ + − − − −	++	12/VII-, SM 0.5 g tw. PAS 0.3 g ev. d.
	10/XI	6.1	No co.					−	+ + + + + −	−	

Group II—*Continued*

No. of cases	Date	Age, years and months	Complaints and remarks	X-ray findings	Mantoux test	BCG-vaccination	Familial contact with Tbc.	Ery. sens.	Antibody against T-P. in serum (Titer of dilution of I.S.C.) 10 20 40 80 160 320	T-P., in serum	Treatment
36	11/X 1958	7.0	Bronc.	No	0×0	−	+	−	+ + + − − −	+	11/X-11/XII, SM 0.5 g tw. PAS 0.3 g ev. d.
	11/XII	7.2	F.					−	+ + + + − −	−	
37	4/XI 1958	7.0	sl. F.	Sh. l.L.R.	10×10	+	+	−	+ + − − − −	++	12/VII-, SM 0.5 g tw. PAS 0.3 g. ev. d.
	8/XII	7.1	No co.					−	+ + + + − −	−	
38	1/IV 1958	8.11	Anor. P. sl. F.	No	10×11	+	−	−	+ − − − − −		1/IV-11/XII, SM 0.5 g tw. PAS 0.3 g ev. d.
	20/X	9.4						−	+ + − − − −	+	
	11/XII	9.6	No co.					−	+ + − − − −	−	
39	1/VIII 1958	8.3	sl. F.	Inc. hil. B.	0×0			−			1/VIII-8/XII, SM 0.5 g tw. PAS 0.3 g ev. d.
	20/X	8.5							+ + + + − −	++	20/X-8/XII, PAS-Na, 2 g, intrav. ev. d.
	8/XII	8.7	No co.					−	+ + + − − −	−	
40	16/IX 1958	10.0	Anor. sl. F.	Sh. l.L.R.	17×12	−	−	−	+ + + + + +	+	16/IX-, SM 0.5 g tw. PAS 0.3 g ev. d.
	24/XI	10.2						−	+ + + + + +	−	
	8/XII	10.3	No co.					−	+ + + + + +	−	
41	30/X 1958	11.0	sl. F.	No	10×8	+	−	−	+ + + + − −	+	30/X-, INH 5mg ev. d. SM 0.5 g ev. d. 4/XI-22/XI, tw, 23/XI-,
	1/XII	11.2	No co.					−	+ + + + + −	−	

A glance over Tables IV and V will suggest that the disappearance of the sensitized erythrocytes (meaning probably the conversion of Type I into Type II) was not so much delayed as might have been expected from the withdrawal of chemotherapy, while the conversion of Type II into Type III was markedly accelerated by the chemotherapy.

Relation between the improvement of types and that of clinical symptoms (Cf. Table VI).

Below will be given the relation in all the cases of Groups I, II, and III, between the improvement of types and that of clinical symptoms. The changes from Type I to Type II or to Type III, or the change from Type II to Type III were taken as signifying an improvement of type of behavior of tuberculin-polysaccharide in blood. As is shown in Table VI, the cases with an improvement in both type and clinical symptoms,

TABLE III

Group III

No. of cases	Date	Age, years and months	Compjaints and remarks	X-ray findings	Mantoux test	BCG-vaccination	Familial contact with Tbc.	Ery. sens.	Antibody against T-P. in serum (Titer of dilution of I.S.C.) 10 20 40 80 160 320	T-P. in serum	Treatment
42	23/X 1958	1.7	sl. F. Diar. C.	Inc. hil. B.	3×3	−	−	+	− − − − − −	++	
	1/XII	1.9	No co.					−	+ + + − − −	++	
43	11/III 1958	2.5	Sw.	Inc. hil. B.	3×3	−	+	+	− − − − − −		
	27/V	2.7						−	+ + − − − −		
	8/XII	3.2	No co.					−	+ + + + − −	−	
44	30/X 1958	4.2	Ano. P.	No	2×2	−	−	+	− − − − − −	++	
	20/XI	4.3						−	+ − − − − −	+	
	11/XII	4.4	No co.					−	+ − − − − −	+	
45	19/IX 1957	5.6	Anor.		13×16	+	+	+	− − − − − −		
	4/II 1958	5.11						−	+ + − − − −		
	6/X	6.6	Bronc.	Inc. hil. B.				−	+ + + − − −	++	
	26/X							−	+ + + − − −	++	
	26/XI	6.7	No co.					−	+ + + − − −	−	
46	11/XI 1958	8.0	Anor.	Sh. R.	24×24	+	+	−	+ + + − − −	+	
	11/XII	8.1	No im					−	+ + + − − −	+	
47	12/XI 1958	9.1	Anor. sl. F.	Inc. hil. B.	3×3	+	−	−	+ + + + − −	++	
	8/XII	9.2	No im.					−	+ + + + − −	++	
48	14/XI 1958	11.0	sl. F. Sw.	No	10×10	+	−	−	+ + + + + −	++	
	11/XII	11.1	No co.					−	+ + + + − −	−	
49	6/X 1958	6.0	Sw. sl. F.	Sh. l. L. R.	4×3	+	+	−	+ + + − − −	+	
	17/XI	6.1	No im.					−	+ + + + + −	++	

were 3 cases (Nos. 43, 45, and 48) out of 8 cases of Group III, 20 cases (Nos. 1–4, 6–7, 9, 11–18, 20 and 22–25) out of 25 cases of Group I, and 13 cases (Nos. 26–31, 33, 35, and 37–41) out of 16 cases of Group II. On the other hand, the cases with no improvement in both type and clinical

TABLE IV

The Term Required for Disappearance of the Sensitized Erythrocytes from Blood: Summary of the cases which were subjected to monthly blood examination

Group	No. of cases	Term required for disappearance of the sensitized erythrocytes	
		Within one month	One-two months
I	11	11	0
III	1	1	0

TABLE V

The Term Required for the Occurrence of Change from Type II to Type III: Summary of the cases which were subjected to monthly blood examination

Group	No. of cases	Term required for occurrence of change from Type II to Type III	
		Within one month	One-two months
II	7	7	0
III	6	2	4

TABLE VI

Relation between Clinical Symptoms and Type of Behavior of Tuberculin-Polysaccharide in Blood

Group	No. of cases examined	No. of cases in which an improvement was found in both type and clinical syptoms	No. of cases in which no improvement was found in both type and clinical symptoms	No. of cases in which an improvement was found only in type, but not in clinical symptoms	No. of cases in which an improvement was found only in clinical symptoms, but not in type
I	25	20	0	5	0
II	16	13	0	3	0
III	8	3	5	0	0

symptoms were 5 cases (Nos. 41, 44, 46–47, and 49) out of 8 cases of Group III, but none in Groups I and II. The cases with type improvement in spite of no improvement of clinical symptoms, were 5 cases (Nos. 5, 8, 10, 19, and 21) of Group I and 3 cases (Nos. 32, 34, and 36) of Group II. This finding will suggest that the improvement of type preceded that of clinical symptoms in certain cases of tuberculosis under chemotherapy (Cf. Table VI).

Generally it may be said that an improvement of type of behavior of tuberculin-polysaccharide in the blood went hand in hand with that of clinical symptoms.

Conclusions

The erythrocytes sensitifized with tuberculin-polysaccharide, which had been identified in the circulating blood of tuberculous infants and children, disappeared generally within one month of the onset of effective chemotherapy.

The tuberculin-polysaccharide, which had been identified only in the serum—that is to say, when tuberculin-polysaccharide was no more identified as being adsorbed to the erythrocytes—, seemed to disappear more quickly in the cases with chemotherapy than in those without it.

An improvement of type of behavior of tuberculin-polysaccharide in the blood in tuberculous infants and children went hand in hand with an improvement of clinical symptoms.

References

1), 2), 3) Arakawa, Oota & Sato, Tohoku J. Exp. Med., 1958, **68**, 93, 99, 105.

4) Arakawa, Oota & Sato, Approximate Estimation of Tuberculin-Polysaccharide in Serum. To be published in this Journal.

5) Arakawa, Oota & Sato, Three Types of Behavior of Tuberculin-Polysaccharide in Blood. To be published in this Journal.

The Tohoku Journal of Experimental Medicine, Vol. 69, No. 4, 1959

On the Morphogenesis of Silicosis

By

Akira Kajita
(梶田 昭)

From the Pathological Laboratory of Prof. K. Akazaki,
Faculty of Medicine, Tohoku University, Sendai

(Received for publication, February 9, 1959)

1. Introduction

Owing to the large increase in autopsy cases in recent years, the patho-anatomical findings of silicosis have come to be well known to pathologists and are offering useful grounds for starting clinical practices as well as hygienic measures.

But we must confess, in answer to the question whether we can fully understand the morphogenesis of this disease by these morphological pictures, that we are not yet in the position to be confident in this respect. The present author[1)2)] has already reported on the results of his patho-anatomico-histological studies on some 30 silicosis cases from Ikuno and Ashio Mines and Tsurumi Foundry, and in the following, plans to summarize these results and at the same time, upon addition of some new ideas, make a general review on the morphogenesis, especially the histogenesis of silicosis.

2. Patho-histology of Simple Silicosis

The unit changes of simple silicosis consist in the silicotic granulomata formed on the wall of the arterioles. These are proliferative foci of histiocytic cells poor in newly formed capillaries, in which soon reticular fibres come into new formation. Whether the histiocytes in charge of the disposition of dusts and the fibroblasts secreting the fibre constituents (glycoproteins as raw material of collagen) are of entirely different origination or these two should be understood as of uniform origin is not yet decided upon.

A full-grown silicotic nodule is a spherical hyaline mass of collagenous fibres, with circular patterns at the periphery and irregular vortical patterns at the center ; sometimes the courses of the fibres at the center betray the course of the blood vessels once situated there.

Upon examination of a hyaline nodule in its earliest-stage formation, it is revealed that, in addition to the cellular-fibrous thickening of the ad-

ventitia, there is always a thickening poor in cellular components in the intima, the elastic fibres between the two layers are disrupted and annihilated and thus a typical hyaline nodule comes into formation. For a thickening of the intima to occur following a compact fibrosis of the adventitia (or the media), in similar processes (e.g., the arteriosclerotic change in syphilitic aortitis) the invasion of plasma protein is emphasised to play an important role by some pathologists, including Büchner[3] and Meyer.[4] I wish to point out here that in the process of irregularly stellar or polygonal silicotic granulomata developing into spherical hyaline nodules, the possibility of diapedesis of plasma protein acting centrifugally from within coming into play is not excluded.

A typical hyaline nodule stands solitarily in the surrounding unaltered alveoles, as a mass of collagen fibre covered by a thin layer of cells, which sends out merely some rudimentary processes along the preexisting connective tissue in the neighborhood, such as the interalveolar and interlobular septa.

In the case when, however, exudative inflammation exists in the alveoles around the nodule, it is found that granulation tissue relatively rich in blood capillaries come to be formed around the nodule and thus the diffuse spreading of the connective tissue fibrils into the exudate results in expansion of the nodule. In such a case, we may say that the inflammation exudate around the nodule offers a field of growth to the granulation tissue and thus induces its expansion.

The silicotic change of the hilar lymph nodes antecedes the change in the pulmonary parenchyma and is severer than the latter. This has been attributed to the accumulation of siliceous dust in the hilar region at an earlier stage. But upon closer examination of a silicotic nodule formed in a lymph node, it may be found that it sends out processes diffusely into the surrounding reticular net, facilitating its fusion with another nodule. Seeing such a picture, I am inclined to conclude that a difference in the anatomical structure rather than that in the quantity of the accumulated dust is the cause of severer silicotic change in the hilar nodes than in the pulmonary parenchyma.

It is a rare finding to observe hyaline nodules forming large coalescent foci by mere juxtaposition. In the formation of the commonly seen coalesced foci, the development of the perinodular granulation tissue, which is determined by the properties of the dust and the phlogogenic factor developed in complication with it as well as the reactive tendency of the individual, plays a fundamental role. It was Giese[5] who has first claimed that when the dust contains much non-siliceous constituents, a granulation tissue more extensive than the hyaline nodule forms the principal change ; he also said that in such cases the hyaline nodule detains the

dust particles in it (according to Gardner[6]), collagen comes into physical or chemical combination with the dust particles here), while the granulation tissue dissolves out these particles to promote the coalescence. According to my experiences, I found a considerable variation in the development of the granulation tissue among the silicosis cases all of whom had experience of working at Ikuno Mine and nowhere else. I can cite some of these as examples of acute silicosis.

3. On Acute Silicosis

On relatively rare occasion, cases of silicosis developed rather rapidly have been reported on (Gerlach & Gander,[7] Terbrüggen & Mohnke,[8] Letterer[9]). One of the cases I have experienced was a man of 41 years who had worked at Ikuno Mine for 10 and a half years and in whom silicosis of Type II developed into a state of widespread coalescene within about two years. Upon autopsy I found that the principal process of this rapid coalescence consisted not in the increase in density or size of the silicotic nodules but in the proliferation of the perinodular granulation tissue. Histologically speaking, a granulation tissue in this case was composed mainly of spindle-cell bundles accompanying infiltrated small round and mononuclear cells and newly formed capillaries; such granulation tissues were found proliferated as if processes were sent out along the interalveolar septa into the surrounding (or so to say in the form of organization of interstitial pneumonia). A part of the fibrocytic bundles running irregular interlacing courses were hyalinized and in some places showed a picture of dense intermixture with the cellular areas. This picture is in good agreement with the picture of granulation tissue decomposing hyaline silicotic tissue advocated by Giese.

Another case of 60 years who had worked in the pits of Ikuno Mine was a case with ascertained silicosis complicated with rheumatoid arthritis before his death. Upon autopsy, a wide-spread focus of silicotic granulation-tissue coalescence very closely similar to that in the preceding case was found in his lung. This reminded me of the remarks of Gough *et al.*[10] who have summarized the Caplan's[11] syndrome under the concept of collagen-type rheumatoid pneumoconiotic changes and that of Baader[12] who postulated a close correlation between silicosis and rheumatoid arthritis and called silicosis "mesencyhmopathia dysproteinotica or silicotica."

Acute silicosis, although named sometimes "Gerüstsklerose" (by Terbrüggen & Mohnke) and sometimes chronic interstitial pneumonia (by Letterer) and described somewhat differently by different authors, consists mostly in increase of the pulmonary stroma due to proliferation of granula-

tion tissues. A specificity in the clinical picture may be found in the excessive burden fulling on the right heart owing to the abrupt rise of resistance in the pulmonary circulation system.

4. Patho-histology of Silicosis Complicated with Tuberculosis

Among the 18 cases of silicosis cases autopsied at Ikuno Mine Hospital since 1955, 10 cases (56%) were found to have complication with tuberculosis. The tuberculous changes found in severe silicosis cases mainly consisted in combination form (tuberculosilicosis) foci, relatively depurated cavities, and old encapsulated caseous foci, freshly disseminated exudative foci being found only in very limited areas in the lung. This was widely different from what I had seen at Ashio Mine and Tsurumi Foundry before 1950, when the autopsied cases nearly always had fresh and wide-spread tuberculous dissemination foci in their lungs. This difference is of course due to the effect of chemotherapy for tuberculosis ; the Ikuno cases had been subjected to treatment with Streptomycin, Para-amino-salicylic acid and Isoniazid for more than one year before their death.

The characteristic feature of tuberculous inflammation consists in coagulation necrosis or caseation. The difference of inflammative reaction developing in coexistence with silicotic tissues in tuberculous cases from the simple granulation tissue formation as in the cases of acute silicosis lies in that in the former case it soon falls into caseation. When the so-called foci of combination form are histologically examined, at a glance we can see that these are tuberculous foci, for they are always extensively caseated and sometimes giant cells of Langhans's type may be found in their peripheral parts. Upon reviewing these foci in silver-impregnated preparations, we find silicotic granulomata on the walls of the arterioles and tuberculous inflammation foci with (exudative) or without (productive) accompanying reconstruction (Umbau) of the alveolar structure contained in one and the same caseous focus, and sometimes a cellular-fibrous wall is formed as if encapsulating it as a whole ; this wall also is often caseated throughout, except a very thin layer on the outmost side. As has been repeatedly noted since the days of Lachmann[13] and Uehlinger,[14] caseous foci grow outwardly, and in the preceding case, caseation invades the peripheral silicotic granulations and even the fibrous capsule, imparting the characteristic of tuberculous foci to the entire focus to the utmost. When the focus has attained an adequately large size, liquefaction and cavity formation may set in and then the new condition of bronchogenic dissemination comes into play.

When the tuberculosis in complication progresses so rapidly as to

decide the fate of the patient single-handed, this may be called the complication form (silicosis with tuberculosis). Such a complication form is reported as comparatively rare in Western countries (Irvine[15] reports that it is more frequent among negroes than among whites), unlike in Japan, but as cited above, the decrease of complication form cases due to the advance in chemotherapy has been observed in my experience at Ikuno Mine too, and it may be inferred that the findings of silicotic tuberculosis cases are drastically changing in Japan too.

5. Summary

Silicosis, as Aschoff[16] has classed it in his text-book, is an "Abkapselnde Entzündung" (encapsulating inflammation) caused by aseptic foreign bodies and represents a mesenchymal defence reaction. In the case when a granuloma is led toward formation of a typical hyaline nodule, the differentiation of the mesenchyma into capillary endothelium seems to be inhibited. The granuloma formed in the arterial wall hampers the nutritional plasma flow and thus causes the formation of the specific hyaline nodule. In this case, whether a detention of dust by collagen is postulated or not, the situation is certainly unfavorable to coalescence and the description Abkapselung (encapsulation) may be rightly applied.

The acute silicosis cases apparently show an exaggerated picture of the formation of coalesced foci, and in such cases, another phlogogenic factor besides SiO_2 seems to have come into play, causing a prosperous proliferation of granulation tissues and the characteristic feature of "encapsulation" above may be said severely injured. When tuberculous infection comes into combination, the caseation of the inflammation granulation brings about a peculiar pathological picture.

References

1) Kajita, A. Kekkaku (Jap.), 1951, **26**, 636.
2) Kajita, A., On the acute development of silicosis, Saishin-Igaku (Jap.). In press.
3) Büchner, F., Spezielle Pathologie, Urban & Schwarzenberg, München & Berlin, 1955.
4) Meyer, W. W., Virchows Arch. path. Anat., 1949, **316**, 268.
5) Giese, W., Verh. Dtsch. Ges. inn. Med., 1936, **48**, 107.
6) Gardner, L. U., Am. J. Path., 1937, **13**, 13.
7) Gerlach, W. & Gander, G., Arch. Gewerbepath., 1932, **3**, 44.
8) Terbrüggen, A. & Mohnke, W., Beitr. path. Anat., 1953, **113**, 44.
9) Letterer, E., Beitr. Klin. Tuberk., 1954, **111**, 501.
10) Gough, J., Rivers, D. & Seal, R.M.F., Thorax, 1955, **10**, 9.
11) Caplan, A., Thorax, 1953, **8**, 29.
12) Baader, E. W., Zschr. Rheumaforsch., 1954, **13**, 258.
13) Lachmann, E., Fortschr. Röntgenstr., 1931, **43**, 407.
14) Uehlinger, E., Beitr. Klin. Tuberk., 1939, **92**, 170.
15) Irvine, L. G., Notes on silicosis, Silicosis, International Labour Office, Geneva, 1940.
16) Aschoff, L., Pathologische Anatomie, Allgemeiner Teil, VIII Aufl., Gustav Fischer, Jena, 1936.

Fig. 1

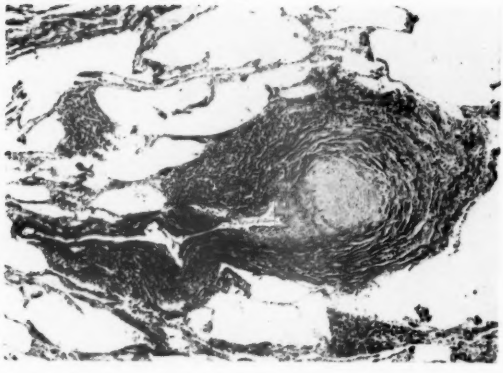


Fig. 2

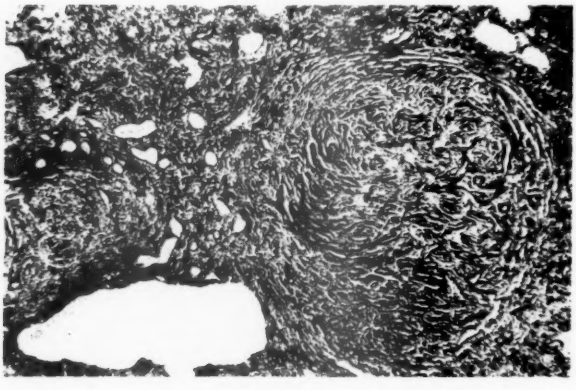


Fig. 3

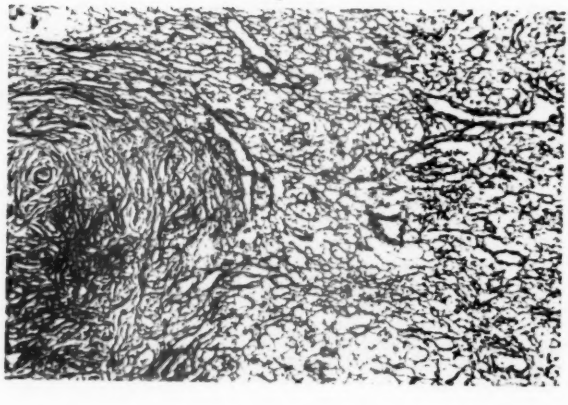


Fig. 4

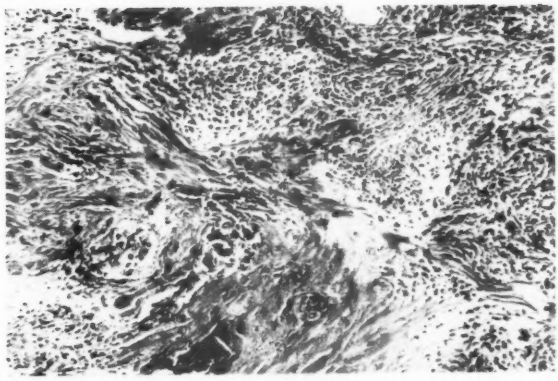


Fig. 5

Fig. 6 (A)

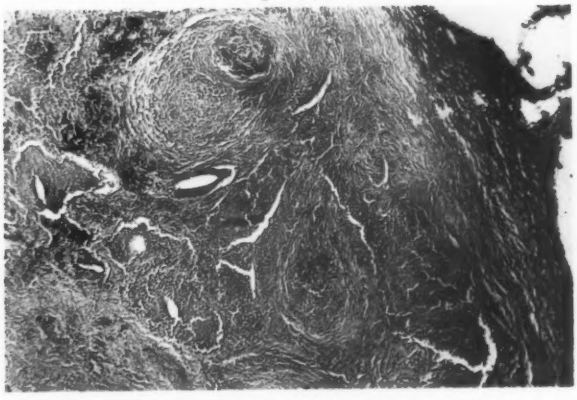


Fig. 6 (B)

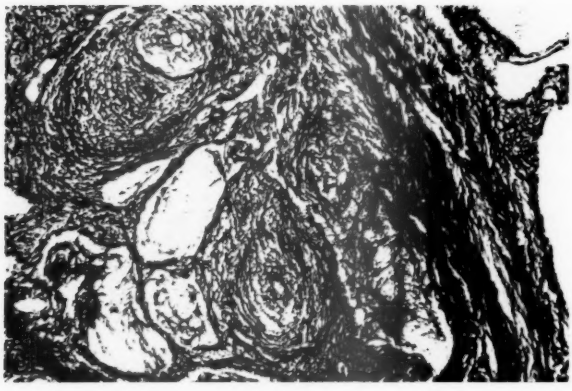


Fig. 1. Silicotic granuloma formed around an arteriole. Coarse collagen fibers already markedly developed in the adventitia. The intima layer is thickened with loose connective tissue. The elastic fibres partly shredded and annuled. As a whole, a hyaline nodule is nearing completion (An Ashio case. Elastica-van Gieson's staining).

Fig. 2. Pneumonic exudation in the alveole around a silicotic nodule, into which diffuse fine connective tissue fibrils run from the periphery of the nodule. Granulation tissues markedly proliferated with numerous capillaries among them (An Ashio case. Azan-Mallory's staining).

Fig. 3. Early stage silicotic change in a hilar lymph node. The nodule is diffusely spreadng over into the surrounding reticular net. (An Ashio case. Silver impregnation).

Fig. 4. Acute silicosis. Broad granulation tissue of cellular-fibrous nature shows a picture of dense intermixture with the hyaline silicotic change. (An Ikuno case. Hematoxylin and eosin staining).

Fig. 5. A case with complicating rheumatoid arthritis. A hyaline silicotic nodule beginning to form around an arteriole in the right part, surrounded by cellular-fibrous granulation tissue extending and proliferating along the interalveolar and interlobular septa on all sides. (An Ikuno case. Hematoxylin and eosin staining).

Fig. 6. A case with complicating tuberculosis. Combination form focus. (A) Hematoxylin and eosin stained. Except a thin capsule seen in the right upper part, the focus is caseated in most part. (B) The same part under silver impregnation. Two silicotic granulomata around an arteriole. A small number of alveolar structures remaining intact. A rather thick fibrous wall covering up the entire focus (A Tsurumi case. Staining as above).

The Tohoku Journal of Experimental Medicine, Vol. 69, No. 4, 1959

Biochemical Studies on Carbohydrates

CCXXVIII. Cornea Mucoid of Suzuki and Keratosulfuric Acid of Meyer School*

By

Sigetosi Kamiyama
(加美山 茂利)

From the Medico-chemical Institute, Tohoku University, Sendai;
Director: Prof. H. Masamune

(Received for publication, February 20, 1959)

In 1939, Suzuki[1] one of Masamune's pupils reported a very noteworthy finding that bovine cornea mucoid contains a sulfated polysaccharide composed of equimolecular acetylglucosamine, galactose and sulfuric acid.[2] Since then, Masamune and his co-workers isolated similar glucidamins, whose carbohydrate moiety is also composed of acetylglucosamine, galactose and sulfuric acid, from chondromucoid[3][4] and osseomucoid[5] and another similar glucidamin from tendon (tendomucoid α)[6].

On the other hand, Woodin[7] treated "mucoids" of cornea with trypsin in 1952 and obtained, he informed, an acid mucopolysaccharide made up of galactose, glucosamine and galactosamine, and in 1953, Meyer and others[8] fractionated cornea after digestion with pepsin and trypsin, and assumed that one of the fractions consisted chiefly of a mucopolysaccharide in which nitrogen, hexosamine, sulfate, acetyl and hexose are apparently present in equimolecular concentrations. The latter authors named the alleged mucopolysaccharide "keratosulfate". Later on, Gardell[9] suspected the presence of a resembling mucopolysaccharide in a protease digestate of nucleus pulposus.

It is a serious problem to decide whether "keratosulfate" was a split product of cornea mucoid by the proteolytic enzymes or is existent as such in cornea, because the protein moiety of cornea mucoid belongs most probably to the class of globulins and is readily digested by pepsin and trypsin like those of the glucidamins from osseomucoid and chondromucoid are.[10] In addition, it is another important problem whether the carbohydrate in cornea mucoid is composed of acetylglucosamine, galactose and sulfuric acid in the molecular proportions of 1:1:0.5 like those in the glucidamins from osseo- and chondromucoid, or in those of 1:1:1 as the

* A great part was read before the 30th General Meeting of Japanese Biochemical Society, Kyoto, July 14–16, 1957.

corresponding carbohydrate in tendomucoid α is.

Therefore, the writer undertook to first fractionate the cornea by a modern procedure without breakdown of its constituents, with the aim of isolating in a pure state of not only the mucoid but also a mucopolysaccharide which resembles keratosulfate, if present, and next separate a mucopolysaccharide from the mucoid after digestion with pepsin and trypsin to compare it with that isolated immediately from cornea extract.

Experimental

Fractionation of Cornea

First stage. Bull corneas were collected, cleaned of adhering tissue, and after washing roughly with physiological saline, stored under 95% ethanol for 2–3 months. They were then scissored into small pieces and dried in a desiccator containing anhydrous calcium chloride. 15 g. of the material thus made ready with 113 corneas were suspended in 2.6 *l.* of distilled water and shaken for 3 days on a machine (preservative thymol), followed by percolation through a gauze and filtration under suction. The tissue residue was treated twice more in a similar manner. The solutions united (slightly turbid) were distilled under reduced pressure to about 250 cc., 320 cc. of glacial acetic acid was added in small portions to pH 1.6 (against TB and CR), and the mixture was stood in a refrigerator (0°C) for several hours. The centrifuged supernatant was set aside in a refrigerator. The centrifugate was taken up in 200 cc. of water by means of 10% potassium carbonate, added in drops to about pH 8.0. The insoluble part was rejected after centrifugation, and again the somewhat cloudy supernatant was precipitated with an equal volume of glacial acetic acid (pH of the mixture 1.5). Centrifuged. The supernatant here was also set aside. The centrifugate was reprecipitated in a similar manner (The centrifuged supernatant here was also set aside), washed with three changes of abs. ethanol and two changes of ether and dried. 180 mg. of a grayish powder was given. It gave the positive reaction not only in Molisch test but also in Neuberg-Saneyoshi and aniline acetate test for hexuronic acid. Biuret positive. It proved inhomogeneous electrophoretically. Lots of crude cornea mucoid amounting to 1.9 g. altogether were obtained after 6 runs of the above procedure.

On the other hand, the three centrifuged supernatant fluids set aside were dialyzed against running water for 4–5 days to remove acetic acid, distilled in vacuo to syrupy consistency and precipitated with excessive ethanol. The precipitate was washed with ethanol and ether and dried in vacuo over anhydrous calcium chloride, giving 331 mg. of a light yellow powder. After several similar runs on new batches of material, 731 mg. in

total of the corresponding powders was obtained—Crude Molisch-positive cornea mucopolysaccharide.

Second stage (application of Zittle and others' procedure[11]). 1.3 g. of the crude cornea mucoid was taken up in 150 cc. of water by addition of the potassium carbonate solution to pH 8.3. The small part remaining undissolved was centrifuged and discarded. 56 g. of ammonium sulfate was added to the slightly cloudy supernatant to half saturation. The deposit was centrifuged off, and additional ammonium sulfate was added to the supernatant here to 3/4 saturation, followed again by centrifugation. To the yellowish, somewhat turbid supernatant (210 cc.) was added 30 cc. of 0.2 *M* borax, prepared by heating and allowed to cool to 30–35°C, in portions, and the mixture was stood in a water-thermostat at 7–8°C for three hours. The abundant precipitate which had subsided was centrifuged off, dissolved in 100 cc. of water and dialyzed against running water for 4 days (preservative toluene). The solution remained nearly clear throughout the dialysis. It was distilled in vacuo at a temperature below 40°C to a syrup, precipitated with excessive ethanol, and after washing with ethanol and ether, dried as above. 850 mg. of a grayish powder was yielded. The procedure was conducted over again, but the product (530 mg.) did not show as yet a single boundary on electrophoresis.

Third stage. 495 mg. of it was dissolved in 60 cc. of water, 0.6 g. of anhydrous sodium acetate was added, and the mixture was chilled on ice and fractionally precipitated with ethanol. However, purification of cornea mucoid in this way looked ineffective so that the fractions, precipitated and dried, were combined (463 mg. altogether; No more precipitate occurred on addition of additoinal ethanol to the solution containing 80% ethanol) to subject to the following fractionation.

Fourth stage. The substance was taken up in 40 cc. of water, shaken with 5 cc. of Dowex 50 (H form) for 20 minutes and filtered with suction with subsequent washing with two 5 cc. portions of water. The filtrate and washing were united and neutralized to pH 5.7 with 1% baryta, whereby slight cloudiness occurred, 1.2 g. of barium acetate was added, and the mixture was centrifuged. The minutely opalescent supernatant was made up to 60 cc. with water, and calculated volumes of ethanol were added stepwise as to raise the ethanol concentration to 20, 25, 35 and 40% (v/v), centrifuging off every deposit on occurrence. The centrifugates were dried. Their respective yields were 20, 194, 38 and 50 mg. Of those fractions, that which had precipitated at 20–25% ethanol proved homogeneous electrophoretically. The whole procedure was repeated with new batches of material leaving out the third stage operation, and the products were combined as pure cornea mucoid—Prep. I.

Fifth stage. The whole product of the crude Molisch-positive cornea

mucopolysaccharide (First stage) (1.06 g.) was suspended in 100 cc. of water, alkalized to pH 8.0 with 10% potassium carbonate and freed from the insoluble part. The solution was neutralized with acetic acid, and after addition of 1.0 g. of barium acetate, fractionally precipitated at 0°C with ethanol similar to above. The yields of the deposits:

Fraction No.	I (water-insoluble)	II	III	IV	V	VI	VII
Ethanol concn. (% (v/v))	0	0–30	30–40	40–50	50–60	60–70	70–80
Yield (mg.)	54	166	360	165	82	106	96

Barium in Fr. II–VII was replaced with sodium for the convenience of immediately analyzying hydrolyzable sulfur as barium sulfate:—Those fractions were separately taken up in 30–50 cc. of water, acidified to pH 2–3 with a small quantity of 1 *N* HCl and dialyzed, followed by shaking with 5 cc. of Dowex 50 (Na form). The mixture was filtered, and the filtrate was distilled in vacuo, precipitated with ethanol containing some anhydrous sodium acetate and dried. Yields here:

Fraction No.	II	III	IV	V	VI	VII
Yield (mg.)	76	265	111	65	66	55

Paper chromatography of their hydrolysates revealed that all those fractions contained galactosamine, glucosamine, galactose, mannose, probable arabinose and xylose and/or lyxose, galactose increasing in the order from V to III and the pentoses in the order from V to VII. Fr. II contained some glucuronic acid in addition to the monosaccharides mentioned.

Sixth stage. To isolate a keratosulfate-like mucopolysaccharide, if present, 229 mg. of Fr. III and 75 mg. of Fr. IV which were rich not only in galactose but also in hydrolyzable sulfur, were put together and fractionated with ethanol at first as a solution in an 0.2 *M* acetate buffer of pH 4.7, with yields:

Fraction No.	I′–V′	VI′	VII′	VIII′	IX′
Ethanol concn. (% (v/v))	0–10 10–20 20–30 30–40 40–50	50–60	60–65	65–70	70–80
Yield (mg.)	0	43	191	21	3

And, then, 255 mg. of VI′–VIII′ combined was again fractionally precipitated with ethanol from a solution, prepared by dissolution in 25 cc. of 1% barium acetate and adjusted to pH 6.8 with baryta. The deposits amounted:

Fraction No.	I″–II″	III″	IV″	V″	VI″	VII″
Ethanol concn.	0–10 10–20	20–30	30–35	35–40	40–45	45–50
Yield (mg.)	0	59	112	99	4	6

By these fractionations, fractions which contained galactose but were devoid of mannose and pentoses could not be separated. Therefore, benzalconium was applied in the next stage.

Seventh stage. 250 mg. of Frs. IV″–V″ was dissolved in 25 cc. of water and adjusted to pH 6.9 with half a drop of 5% NaOH, and 5 cc. of 10% benzalconium chloride (" Osvan solution " Takeda) was added, whereby a flocculent precipitate occurred. The mixture was placed in a refrigerator for 2 hours with occasional agitation. The precipitate was centrifuged off, washed with 2 changes of chilled 1% benzalconium chloride and extracted with four changes of ethanol. On additing 1.2 g. of anhydrous sodium acetate to the somewhat opalescent solutions united (80 cc.), a gelatinous deposit appeared. After standing in the refrigerator overnight, the jelly was centrifuged off, washed twice with 25 cc. of ethanol, which was half-saturated with anhydrous sodium acetate, twice with plain ethanol and once with ether and dried. Yield, 169 mg. A 164 mg. portion was subjected to a repetition of the treatment with benzalconium chloride, yielding 104 mg. of a substance.

Eighth stage. A solution of 100 mg. of the product above was taken up in 20 cc. of water and again fractionated with ethanol in the presence of 200 mg. of barium acetate for caution's sake.

The yields of fractions:

Fraction No.	I‴–II‴	III‴	IV‴	V‴	VI‴	VII‴
Ethanol concn. (% (v/v))	0–10 10–20	20–30	30–35	35–40	40–45	45–50
Yield (mg.)	0	5.5	76.5	18.0	7.0	0

Frs. IV‴–VI‴ were dissolved in 30 cc. of water and changed into sodium salt by shaking with Dowex 50 (Na form) as above. The filtrate was condensed, precipitated with ethanol containing sodium chloride, and after washing with ethanol and ether, dried in vacuo to a constant weight over anhydrous calcium chloride—Prep. II. 91 mg. was the yield of a grayish powder given—Pure Molisch-positive Cornea Mucopolysaccharide.

Properties of the Preparations of the Mucoid and of the Molisch-positive Mucopolysaccharide in Cornea (Prep. I and II)

Prep. I

1) *Electrophoresis.* A Tiselius apparatus of HTD-1 type Hitachi was used. The patterns are shown in Fig. 1.

2) *Test-tube tests.* Molisch, biuret and the test of hydrolyzable sulfur as $BaSO_4$ positive; Neuberg-Saneyoshi, Goldschmiedt and aniline acetate test all negative.

3) *Paper chromatography of the components.* a) Sugars. 10 mg. of the

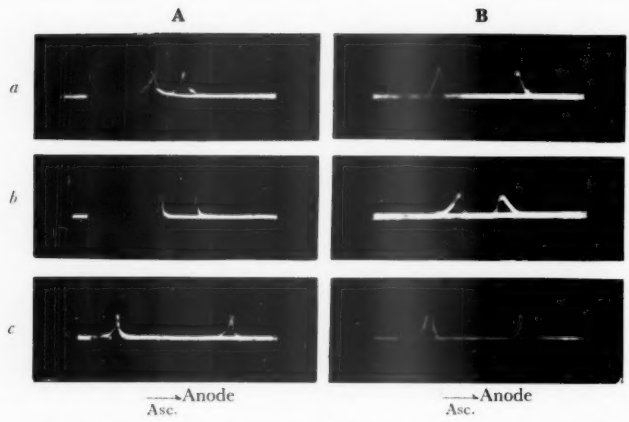


Fig. 1. Electrophoresis patterns (×2/3) of 1% solutions in veronal-HCl buffer, pH 8.6 and I 0.2 (*a*), acetate buffer, pH 4.7 and I 0.2 (*b*) and glycine-HCl buffer, pH 2.0 and I 0.2 (*c*) of Prep. I (**A**) and II (**B**); temperature 15°C. Mobility:

Prep. I pH 8.6 $\frac{(-1.70)\times 0.01182\times 0.18}{30\times 60\times 0.005} = -0.02\times 10^{-4}$ cm²/volt/sec (5 mA, 30 min.)

pH 4.7 $\frac{(-1.30)\times 0.00567\times 0.3}{40\times 60\times 0.008} = -1.15\times 10^{-4}$ " (8 mA, 40 min.)

pH 2.0 $\frac{(-0.70)\times 0.01810\times 0.3}{120\times 60\times 0.010} = -5.28\times 10^{-5}$ " (10 mA, 120 min.)

Prep. II pH 8.6 $\frac{(-1.15)\times 0.01182\times 0.18}{30\times 60\times 0.005} = -2.72\times 10^{-4}$ " (5 mA, 30 min.)

pH 4.7 $\frac{(-1.55)\times 0.00567\times 0.18}{30\times 60\times 0.005} = -1.76\times 10^{-4}$ " (5 mA, 30 min.)

pH 2.0 $\frac{(-0.70)\times 0.01810\times 0.18}{60\times 60\times 0.005} = -1.27\times 10^{-4}$ " (5 mA, 60 min.)

preparation was hydrolyzed with 1 *N* H_2SO_4 by heating at 100°C for 5 hours in a sealed tube. The hydrolysate was neutralized with about 2.5 cc. of the satd. baryta to pH 5.9, and the centrifugate was washed with warm water. The supernatant and washing were passed through a column of Dowex 50 (H form; 2 cc.) and washed with water. The column was then washed with 20 cc. of 1 *N* HCl, and the eluate was distilled at a temperature below 40°C to dryness and freed from the hydrochloric acid as far as possible by repeated vacuum-distillations with water. The final dry still-residue here will be referred to as Residue *a*. The effluent through the column and washing were also distilled in vacuo to dryness—Residue *b*.

Next a 10×60 cm. paper sliver (Toyo Roshi filter paper No. 2) was spotted with a 1/5 portion of the still-residues *a* and *b* and irrigated with solvent butanol-pyridine-water (5:3:2 by volume) according to Masa-

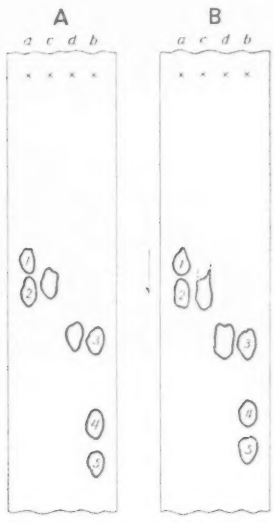


Fig. 2. Sugar paper chromatograms (×1/3) of the hydrolysates of Prep. I (**A**) and II (**B**); irrigation descending; temperature of test 22–25°C; duration of test 20 hrs. Reference runs effected with 0.01 cc. of 1% solutions with respect to indivisual sugars.

a, b: reference. c: Residue *a*. d: Residue *b*. 1 galactosamine, 2 glucosamine, 3 galactose, 4 mannose, 5 xylose.

mune and Yosizawa[12]. Indicator aniline hydrogen phthalate reagent of Partridge. See Fig. 2. Two spots were shown at the positions corresponding to glucosamine and galactose.

b) Amino acids. 5 mg. of the substance was heated with 1 cc. of 6 *N* HCl in a sealed tube at 100°C for 24 hours. The hydrolysate was evaporated in vacuo over solid caustic soda to dryness. The still-residue was repeatedly evaporated to dryness with water further on. The final still-residue which was nearly free from the free hydrochloric acid was dissolved in 0.2 cc. (The solution contained some humin), and 0.04 cc. of the solution corresponding to 1 mg. of the unhydrolyzed substance was sampled on a 30×30 cm. sliver of Toyo Roshi filter paper No. 50 to develop a two-dimensional chromatogram, effecting the primary irrigation with mixture butanol-acetic acid-water (4:1:2) and the secondary with 80% phenol in an atmosphere containing ammonia. Temperature of test 23–25°C. Spray reagent 0.2% ninhydrin in moist butanol. See Fig. 3. Spots were indicated at the position corresponding to aspartic and glutamic acids, serine, glycine, threonine, hexosamine, alanine, tyrosine, valine

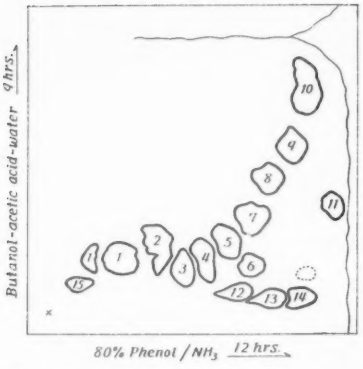


Fig. 3. Two-dimensional amino acid-paper chromatogram (×1/3) of the hodrolysate of Prep. I.

1 aspartic acid, 2 glutamic acid, 3 serine, 4 glycine, 5 threonine, 6 hexosamine, 7 alanine, 8 tyrosine, 9 valine and/or methionine, 10 phenylalanine, leucine and/or isoleucine, 11 proline, 12 histidine, 13 lysine, 14 arginine, 15 a peptide.

and/or methionine, phenylalanine, leucine and/or isoleucine, proline, histidine, lysine, arginine and a peptide.

4) Quantitative analyses. The analytical figures obtained are shown in Table I. The molecular ratios 1 : 1 : 0.5 of acetylglucosamine, galactose and

TABLE I

Composition of the Preparations

Analysis	In per cent			In molecular proportion**		
	Prep. I	Prep. I′	Prep. II	Prep. I	Prep. I′	Prep. II
N*	11.24	5.31	4.27	8.36	1.99	1.79
Glucosamine†	17.25	34.10	30.52	1.00	1.00	1.00
Galactose‡	15.43	32.10	30.41	0.90	0.91	0.99
Acetyl§	,4.70	8.25	6.68	1.13	1.01	0.91
Hydrolyzable sulfur//	1.77	3.46	5.25	0.58	0.57	0.96
Ash¶	9.27	9.63	10.90			

* Micro-Kjeldahl. † Masamune and Yosizawa.[13] ‡ Masamune and Sakamoto[14]. § Suzuki's modification of Friedrich, Rapoport and Sternberg method[15]. // Suzuki[15]. ¶ Pregl method without use of H_2SO_4. ** Glucosamine was designated 1.

sulfuric acid agree with those of the same components in the glucidamins from chondro-[3)4)] and osseomucoid[5)].

5) Optical rotation. 17.2 mg. of the mucoid was taken up in 1.72 cc. of water and adjusted to pH 6.9 with 0.01 cc. of 0.1 *N* NaOH.

$$[\alpha]_D^s = \frac{-0.31 \times 100}{0.984 \times 1} = -31.5°$$

6) Ultraviolet spectra. A 2 mg% solution of the substance (pH 7.2) was examined in a photoelectric spectrophotometer of EPU-2A type Hitachi. The maximum absorption took place at wave-lengths 278–280 mμ.

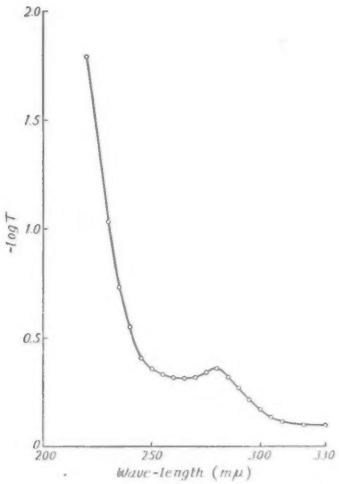


Fig. 4. Ultraviolet absorption spectrum of a 2 mg% aqueous solution (pH 7.2) of Prep. I.

7) Separation of the carbohydrate moiety after digestion with pepsin and trypsin and its properties. 200 mg. of the mucoid was suspended in 20 cc. of water and dissolved by adding 5% NaOH to pH 8.5. The solution was incubated at first with 4 mg. of pepsin (Tokyo Kasei) at 37°C for 3 days after adjustment with HCl to pH 2.0 and afterwards with 4 mg. of crystalline tryspin (Mochida) for 2 days after adjustment with NaOH to pH 8.0, continuing each digestion until no more shift of hydrogen ion concentration occurred. The digestate was neutralized to pH 6.9 with 10% hydrochloric acid, and 125 mg. of calcium acetate was added and 0.075 cc. of glacial acetic acid to pH 4.0. The mixture was precipitated with 28 cc. (1.4 volumes) of 99% ethanol, stood for one day in a refrigerator and centrifuged off in a refrigerated centrifuge. The centrifugate was washed with ethanol and ether and dried in vacuo over anhydrous calcium chloride. 95 mg. of the white powder given was shaken with two 10 cc. portions of

90% (v/v) phenol, and the substance that had changed gelatinous was washed with ethanol three times and with ether two times and dried as above—Prep. I'. Yield 76 mg. Molisch strongly positive; hydrolyzable sulfur detected; biuret faintly positive; Neuberg-Saneyoshi and aniline acetate test negative. The molecular proportions of glucosamine, galactose and sulfuric acid coincided with those encountered in cornea mucoid itself (Table I).

Prep. II

1) Homogeneity. Electrophoretic patterns which revealed that the preparation was homogeneous are shown in Fig. 1.

2) Test-tube tests. Molisch strongly positive; hydrolyzable sulfur was detected as barium sulfate; biuret test and Goldschmiedt, Neuberg-Saneyoshi and aniline acetate test for hexuronic acid all negative.

3) Paper chromatography of the sugar components. The paper chromatoaraphy was carried out as in the case of cornea mucoid. The chromatogram developed exhibited two spots at the positions corresponding to glucosamine and galactose. See Fig. 2.

4) Quantitative analyses. See Table I. Unexpectedly the molecular ratios of glucosamine, galactose and sulfuric acid were found to be 1:1:1 contrary to the corresponding ratios in cornea mucoid which are 1:1:0.5.

5) Optical rotation.

$$[\alpha]_D^3(H_2O)=\frac{(-0.05)\times 100}{1\times 0.99}=-5.1^\circ$$

Discussion

The experimental data have established that there are both a glucidamin and a Molisch-positive mucopolysaccharide in cornea. The former which is Suzuki's cornea mucoid contains a carbohydrate composed of acetylglucosamine, galactose and a sulfuric acid in the molecular proportions of 1:1:0.5, and the latter an also sulfated polysaccharide which is similar to the one in the mucoid regarding kinds of component but is different quantitative-analytically, the molecular proportions of those components being 1:1:1. Thus it is clear that Meyer and others' preparation of "keratosulfate" was a mixture of the mucopolysaccharide produced by digestion of cornea mucoid and the mucopolysaccharide originally existent as free in cornea. It might be further stated that the carbohydrates in the glucidamins from chondro- and osseomucoid and that in cornea mucoid on the one hand, and those in tendomucoid and the free mucopolysaccharide in cornea above on the other are the same respectively at least in quantitative composition. Whether the mucopolysaccharide Gardell assumed in nucleus pulposus is present as such in the tissue or was derived from a glucidamin by proteolytic enzymes awaits a study.

SUMMARY

1. Suzuki's mucoid and a mucopolysaccharide made up chiefly of acetylglucosamine, galactose and sulfuric acid were isolated in an electrophoretically pure state from bull cornea and examined physically and chemically.

2. Analyses disclosed that the carbohydrate moiety of the mucoid is composed of acetylglucosamine, galactose and sulfuric acid in the molecular proportions of 1:1:0.5, which was substantiated on the moiety separated after digestion with pepsin and trypsin of the mucoid.

3. The mucopolysaccharide existing as a chemical entity in the cornea contains acetylglucosamine, galactose and sulfuric acid in the molecular proportions of 1:1:1.

4. The glucidamins from chondro- and osseomucoid and corneamucoid contain most probably the same carbohydrate in the molecule.

5. It is concluded that Meyer's preparation of " keratosulfate " was a mixture of the mucopolysaccharide as a digestion product of cornea mucoid and the above free mucopolysaccharide in cornea, which differ from each other in hydrolyzable sulfur content as expressed in molecular proportion to acetylglucosamine or galactose.

The expenses of this work were defrayed by a grant given from the Ministry of Education through the Grant Committee for Medical, Dental and Pharmaceutical Researches. H. Masamune.

References

1) Suzuki, J. Bioch. (Japan), 1939, **30**, 185.
2) Later on, Masamune, Yosizawa and Maki (Tohoku J. Exp. Med., 1951, **53**, 237) reexamined Suzuki's specimen and suggested that the mucoid was contaminated with a polysaccharide or polysaccharides containing galactosamine and glucuronic acid.
3) Hiyama, Tohoku J. Exp. Med., 1949, **51**, 65.
4) Masamune & Yasuoka, *ibid.*, 1947, **49**, 181.
5) Hiyama, *ibid.*, 1949, **51**, 97.
6) Sasaki, *ibid.*, 1945, **48**, 307.
7) Woodin, Bioch. J., 1952, **51**, 319.
8) Meyer, Linker, Davidson & Weissmann, J. Biol. Chem., 1953, **205**, 611.
9) Gardell & Rastgeldi, Acta chem. Scand., 1954, **8**, 362.
10) Asagi, Tohoku J. Exp. Med., 1949, **50**, 95.
11) Zittle, Smith & Krejci, Arch. Biochem., 1948, **19**, 9.
12) Masamune & Yosizawa, Tohoku J. Exp. Med., 1953, **59**, 1.
13) Masamune & Yosizawa, *ibid.*, 1956, **64**, 53.
14) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
15) Suzuki, J. Bioch. (Japan), 1938, **27**, 367.

Vision Quest

Book Two

Errata

H. Masamune, S. Hakomori, H. Kaketa and T. Sugo, Urinary Mucopolypeptides and Mucoproteins in Cancer, I. Differences in Polarographic Behaviour of Urinary Mucoproteins of Patients, Cancerous and Inflammatory, and of Normals. This Journal, Vol. 69, No. 4, p, 371, 1959.

Read *V* for any of the *mV*'s in the text, Figs. 1–6, Table I and *mA*'s in Fig. 7.

The Tohoku Journal of Experimental Medicine. Vol. 69, No. 4, 1959

Urinary Mucopolypeptides and Mucoproteins in Cancer

I. Differences in Polarographic Behaviour of Urinary Mucoproteins of Patients, Cancerous and Inflammatory, and of Normals*

By

Hajime Masamune, Sen-itiroh Hakomori, Hirosi Kaketa
(正宗 一) (箱守 仙一郎) (掛田 博)
and **Takasi Sugo**
(須郷 昴)

From the Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, February 20, 1959)

In his well-known article which appeared in 1938 " Über diagnostisch verwendbare Veränderungen im Serum bei Krebs ", Waldschmidt-Leitz[1] pointed out the diminished sulfhydryl-activity of cancerous serum as compared with that of normal serum, being based on the weakened re-activating potency of cancerous sera on inactivated cathepsin or papain, and utilized it for the diagnosis of cancer, determining that potency of sera on liver methylglyoxalase an SH enzyme. Nevertheless, its availability for the practical purpose was soon negated by Meyer-Heck[2] in the next year. At that time, the polarographic method of Heyrovský and Shikata was being increasingly appreciated regarding not only inorganic but also organic compounds provided with redox potential. And Brdička[3] found " protein wave " and ascribed it mainly to SH- and S-S-bearing amino acids (cystine and cysteine) in the protein molecule. Its application for diagnosis of cancer was attempted as a natural result. According to him,[4] the decreased pre-natrium wave in dilute NH_4OH or decreased catalytic wave in the cobalt buffer of denatured sera and the increased catalytic wave of the deproteinized sera in the cobalt buffer are signs of cancer.

A number of workers have been engaged in studying " cancer wave " (Brdička), of whom Müller,[5] Winzler,[6] Sasai[7] and Wada[8][9] are celebrated. They agreed in concluding that neither the decreased pre-natrium wave nor the increased filtrate wave is specific to cancer, because those waves occur also in tuberculosis, rheumatic fever and many other inflammatory diseases. Regarding further attempts to distinguish the wave

* Read before the 31st Annual Meeting of the Japanese Biochemical Society, Sapporo, July 14–16, 1958. 229th report of Masamune and co-workers' " Biochemical Studies on Carbohydrates."

in cancer from those in the other diseases, one will first remind of the early work of Waldschmidt-Leitz.[10] He described to have been able to separate the cancer-specific " polarographically active factor " (PAF) from other PAFs in the filtrate of cancerous sera by simple ethanolic or sulfosalicylic acid fractionation. This finding was denied by Sato[11], who extended the idea of Waldschmidt-Leitz, putting forward " S.M. index " ratio in height of the wave at -1.6 ■V given by the sulfosalicylic acid filtrate to that by the 60% methanol filtrate. He said that the ratio amounts to 2.5 for cancer, and to 1.2 for inflammation. As a similar trial, Müller and Davis[12] proposed " protein index " height ratio of the direct catalytic wave to the filtrate wave to find a characteristic value in cancer and in inflammation. However, it was later on disclosed that distribution of the waves of different heights among the fractions depends largely upon the relative concentrations of albumin and lipoproteins in sera. On the other hand, it was long said that a mucoprotein or mucoproteins in sera may play a role as PAFs, so that Mayer[13] pursued the PAFs and obtained a larger amount of mucoids after ethanolic fractionation of the sulfosalicylic acid filtrate of cancerous sera than from the corresponding fraction of normal sera, and compared their cystine contents with each other, but what embarrassed him was that cystine was found more in the normal mucoid than in the cancerous. Winzler and Burk[6] reported " polypeptidemia " in cancer, whereas, after scrutinies, they became aware that the polypeptidemia signifies nothing more than an elevated glycoprotein level in sera.[14] Similarly Sasai[15] obtained a mucoprotein adsorbable to diatomaceous earth as the major PAF in sera. A question is still left, however, whether the increased polarographic activity reflects a mere elevated mucoprotein level or increase of a mucoprotein having a specific structure, which causes the heightened polarographic wave. The latter possibility has become plausible because a series of recent works[16)–22] in this laboratory demonstrated structural deviation of mucopolysaccharide and mucoproteins of cancerous tissues or cancerous body fluids. This account deals with experiments to see qualitative differences in polarographic behaviour of various mucoproteins as a whole of cancerous, inflammatory and normal urines and to fractionate whole urine followed by polarography of the fractions separated.

Experimental

Methods

Materials

Kaketa fraction[17] mucoprotein fraction adsorbable to benzoic acid was separated from various urines, normal and pathological. The cancerous urinary toxohormone without KIK potency and the corresponding

component of normal urine were prepared in an electrophoretically pure state from the above fractions according to Masamune *et al.*[16] and urinary KIK-factor which is also toxohormone active and the corresponding fraction of normal urine according to Kaketa[17].

Polarography

The patterns were taken in a polarograph of Type SH Shimazu, after adjustment of sensitivity of the shunt (*S*) to 1/150. Murcury was dropped in the rate of one drop per 3 seconds. Room temperature 20°C. The buffer contained 10^{-4} *M* hexammine cobaltic chloride, 0.8 *N* $NH_4(OH)$ and 0.1 *M* NH_4Cl, namely, 1 cc. of 10^{-3} *M* hexammine cobaltic chloride (267.5 mg. of $Co(NH_3)_6Cl_3$ in 1 *l.* of water) was mixed with 8 cc. of 1 *N* NH_4OH and 1 cc. of 1 *M* NH_4Cl. Of the solutions to be examined, 1 cc. each containing 300–800 γ of a substance was mixed with 5 cc. of the above buffer and subjected to polarography.

Preparation of derivatives of the Kaketa fractions

a) Reaction with monoiodoacetic acid was let occur according to Dickens.[23] 50 mg. of the material was dissolved in 1 cc. of 0.01 *N* NaOH (pH of the solution neutral), mixed with 1 cc. of 0.1 *M* sodium bicarbonate and 0.2 cc. of 0.1 *M* potassium monoiodoacetate and stood for 2 hours at room temperature. After two days' dialysis against running water, it was distilled in vacuo to a small volume, precipitated with ethanol and dried in a desiccator ($CaCl_2$). 30 mg. was the yield in averages.

b) Treatment with formaldehyde in dilute alkali. Since methylol attached to the amino or imino group is liberated by dialysis, the reaction mixture after treatment with formaldehyde is used as such:—

Two 500 γ portions of the cancerous mucoprotein fraction adsorbable to benzoic acid were dissolved each in 0.5 cc. of 1 *N* NaOH, 0.5 cc. of 10% formaldehyde was added to one of the solutions and the same volume of water to the other, and after standing at room temperature for 30 minutes, the mixtures were further mixed with 5 cc. of buffer (10^{-4} *M* $Co(NH_3)_6Cl_3$)-(0.8 *N* NH_4OH)-(0.1 *M* NH_4Cl) to effect polarography. Two 500 γ portions of the corresponding normal mucoprotein fraction were also treated similar to above.

c) Oxidation with iodine in dilute sodium carbonate (hypoiodite) was processed according to McLeod and Robison.[24] Then the reaction mixture was dialyzed against running water and further treated as in a). Yielded was 40 mg. of the product from 50.2 mg. of the material in one case.

d) Coupling of the fraction with diazobenzenesulfonyl was made after Landsteiner[25], using diazobenzenesulfonylchloride and sodium carbonate. The reaction mixture here also was dialyzed, condensed, precipitated and dried as in a). The materials 50 mg. Mean yield of the products 45 mg.

Fractionation of urinary mucoproteins by anion-exchange chromatography

Whole urine was fractionated by the aid of Dowex 1×2 an anion exchanger.[26] In advance, the resin was regenerated from the commercial chloride with 2 *N* NaOH and converted into the borate form by repeated washings with 0.1 *M* borax-HCl buffer of pH 8.6 (Dissolve 25.222 g. of $Na_2B_4O_2 \cdot 10H_2O$ in 340 cc. of 0.2 *N* HCl and fill up to 1 *l.* with H_2O). It was poured into a 1.3 cm. diameter tube as high as 15 cm. and washed with an 8 times dilution of the 0.1 *M* borax-HCl buffer above (0.0125 *M*). 20 cc. of the urine, adjusted to sp. gr. 1.015 and alkalized to pH 8.5 with 1 *N* NaOH, was added on the column and sucked slowly. The column was then washed with 50 cc. portions of 0.0125 and 0.025 *M* borax-HCl buffer of pH 8.6, 80 cc. of 0.05 *M* borax-HCl buffer of the same pH, 150 cc. of 0.1 *M* borax-HCl buffer of the same pH and 150 cc. portions of 0.1 *M* acetate buffer of pH 3.0, 0.1 *N* acetic acid and 0.1 *N* HCl in sequence. The effluent was collected in 10 cc. fractions automatically. And, of each fraction, 1 cc. was diluted with 3 cc. of water to measure the ultraviolet absorption at 280 mμ, and another 1 cc. portion was analyzed by a quantitative Molisch method.[27] Five main peaks A, B, C, D and E were recognized. Fractions forming the corresponding peaks were united together and evaporated in vacuo to dryness. Without regard to their high salt concentration, the residues were each dissolved immediately in 10 cc. of the cobalt buffer above for polarography.

Results and Comment

1) Polarographic behaviour of the fraction adsorbable to benzoic acid

The waves of this fraction of cancerous, inflammatory and normal urines are shown in Fig. 1 and illustrated in Table I.

When the polarograms of the same amounts of the substances, which had been obtained from different urines and dissolved in the same volumes of the buffer, are compared with one another, it is noticed that the catalytic wave at −1.2∼−1.3 ■V corresponding to the maximum reducing wave of $Co^{++} \rightarrow Co$ is strongly depressed and that at −1.5∼−1.6 ■V becomes extremely remarkable in most cases of cancerous urines and in some cases of inflammatory urines, while the former wave is pronounced in most cases of normal urines, in contrast with the lower latter. Thus the height ratio of the wave at about −1.2 ■V to that at about −1.6 ■V exceeds 2.0 in most cancer cases and in some inflammation cases, but is below 0.3 in most normal cases. Although the rule can not always hold, it is definitive that a certain qualitative difference exists between the benzoic acid-adsorbable mucoprotein fraction of cancerous and inflammatory urines on one side and that of normal urines on the other. As is described even in the current literature, the elevated polarographic wave in cancerous

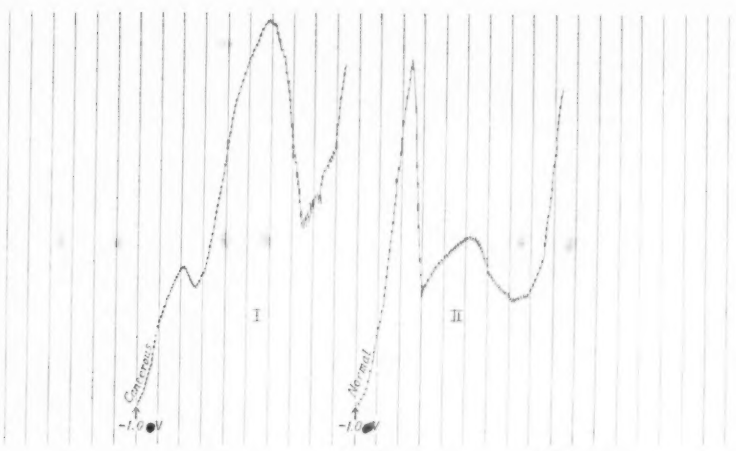


Fig. 1. Polarograms of the fractions adsorbable to benzoic acid of pooled cancerous urine (I) and of pooled normal urine (II). 840 γ of each fraction was dissolved in 6 cc. of buffer (10^{-4} *M* $Co(NH_3)_6Cl_3$)-(0.8 *N* NH_4OH)-(0.1 *M* NH_4Cl). Note the low wave at about −1.2 ■V (*a*) and the pronounced wave at about −1.6 ■V (*b*) in case of the cancerous fraction and the wave heights in inverse relationship in the case of normal fraction. The ratio of wave heights *b*/*a* is 2.7 for I and 0.52 for II.

sera has been attributed to the increased level of PAFs, but, the possible polarographically active structure has never before been considered seriously.

2) Waves of pure urinary mucoproteins

The mentioned inclination of waves regarding Kaketa fractions was also recognized in the KIK-inactive toxohormone and toxohormone-active KIK-factor from cancerous urine and the corresponding components of normal urine. See Fig. 2 and 3. The height ratio of the wave at −1.25 ■V to that at −1.58 ■V is calculated at 1.31 in the cancerous KIK-inactive toxohormone, but the corresponding ratio in the corresponding normal substance at 0.65. Similarly, the ratio of the KIK factor was found to be 1.17 and that of the corresponding normal substance to be 0.65.

The ratio is much lower in the both pure mucoproteins than in the benzoic acid-adsorbable mucoprotein fraction, suggesting that there exists an unknown substance, which characterizes the polarographic waves of cancerous Kaketa fraction, and that it is eliminated during preparation of the pure mucoproteins.

3) Waves of Kaketa fractions treated with various reagents

TABLE I

Heights in cm. of Waves at −1.2 and −1.6 ■V and Their Ratio of the Urinary Mucoprotein Fraction Adsorbable to Benzoic Acid (500 γ) in Cancer, Inflammation and Normal

Cancer				Inflammation and other diseases				Normal			
Case	Height of wave		Ratio $\frac{b}{a}$	Case	Height of wave		Ratio $\frac{b}{a}$	Height of wave		Ratio $\frac{b}{a}$
	at −1.2 ■V (a)	at −1.6 ■V (b)			at −1.2 ■V (a)	at −1.6 ■V (b)		at −1.2 ■V (a)	at −1.6 ■V (b)	
Gastric cancer	1.2	4.8	4.0	Tuberculosis (exudative)	3.1	2.5	0.80	7.8	2.5	0.32
Gastric cancer	0.5	5.5	11.0	Tuberculosis (productive)	4.5	3.8	0.83	8.5	1.8	0.21
Esophagus cancer	2.3	5.8	2.5	Tonsillitis	0.6	5.2	8.6	7.8	2.3	0.29
Gastric cancer with ascites	0.8	6.2	7.7	Gangrene of lung	4.3	2.2	0.51	7.2	2.8	0.38
Breast cancer	9.8	1.5	0.15	Common cold	4.2	1.5	0.35			
Uterus cancer (4th grade)	2.2	6.0	2.7	Myositis	3.3	2.5	0.75			
Hepatoma	1.3	4.9	3.7	Pleuritis	1.2	5.8	4.8			
Gastric cancer	1.2	4.7	3.9							
Mediastinal tumor	3.5	4.0	1.1							
Uterus cancer (2nd grade)	7.6	2.2	0.27							

a) When treated with potassium monoiodoacetate and weak alkali, the wave at about −1.6 ■V of the fraction heightens in cancer and lowers in inflammation and normal (See Fig. 4 and 6 (Ic, IIc)).

b) Formaldehyde treatment in dilute alkali gives rise to decrease of the wave at about −1.6 ■V in cancer but less pronounced decrease of the same wave in normal, wherein remarkable deformation of the waves occurs (See Fig. 5).

c) Oxidation with hypoiodite also decreases markedly the wave at −1.6 ■V to give a flatt wave in cancer and more pronouncedly in normal (See Fig. 6, Ib and IIb).

d) No change of peaks is caused by coupling with diazobenzenesulfonyl (Fig. 6, Id and IId).

It aroused our interest that the cancerous and normal Kaketa-fractions behave oppositely before and after treatment with monoiodoacetate when polarographed. For explanation of this phenomenon one could assume that, in the cancerous substance, a certain structure bearing S-S links is turned polarographically much more active by introducing the

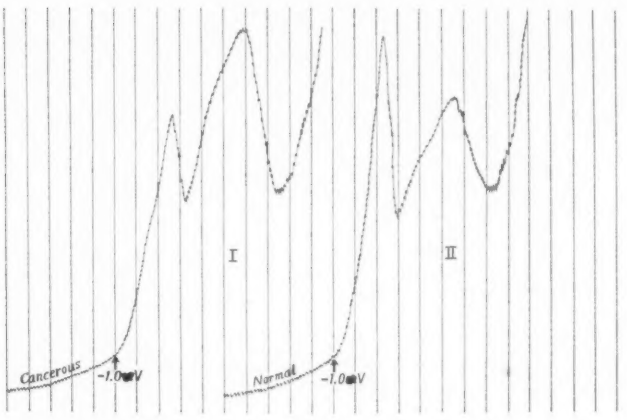


Fig. 2. Polarograms of KIK-inactive toxohormone from cancerous urine (I) and of the corresponding fraction of normal urine (II). 101 γ of I and 102 γ of II were dissolved each in 6 cc. of buffer (10^{-4} *M* $Co(NH_3)_6Cl_3$)-(0.8 *N* NH_4OH)-(0.1 *M* NH_4Cl). Note the same relation as to the two wave-heights (*a* and *b*) as in Fig. 1. The ratio *b*/*a* is 1.31 for I and 0.65 for II.

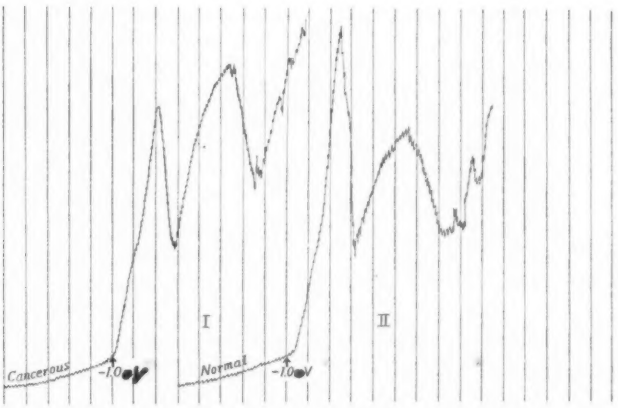


Fig. 3. Polarograms of toxohormone-active KIK-factor from cancerous urine (I) and of the corresponding component of normal urine (II). 230 γ of I and 275 γ of II were each dissolved in 6 cc. of buffer (10^{-4} *M* $Co(NH_3)_6Cl_3$)-(0.8 *N* NH_4OH)-(0.1 *M* NH_4Cl). Note the same relation between the two wave-heights as in Fig. 1. The ratio of wave heights *b*/*a* is 1.17 for I and 0.65 for II.

carboxy-thiomethyl-ether group.

4) *Comparison of polarographic activities of fractions separated by anion*

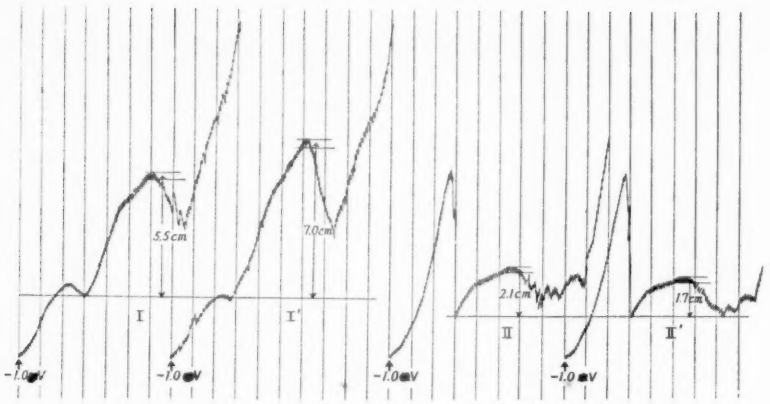


Fig. 4. Polarograms of the cancerous mucoprotein fraction adsorbable to benzoic acid (I), the corresponding normal fraction (II) and their carboxymethylthio-ether derivatives (I′ and II′). 500 γ of each specimen was dissolved in 6 cc. of buffer (10^{-4} *M* $Co(NH_3)_6Cl_3$)-(0.8 *N* NH_4OH)-(0.1 *M* NH_4Cl). Note that the wave height of the cancerous fraction is increased from 5.5 to 7.0 cm. by treatment with potassium monoiodoacetate and alkali, whereas the wave height of the normal fraction is decreased from 2.1 to 1.7 cm. by the same treatment. The polarograms in cases of inflammation are omitted.

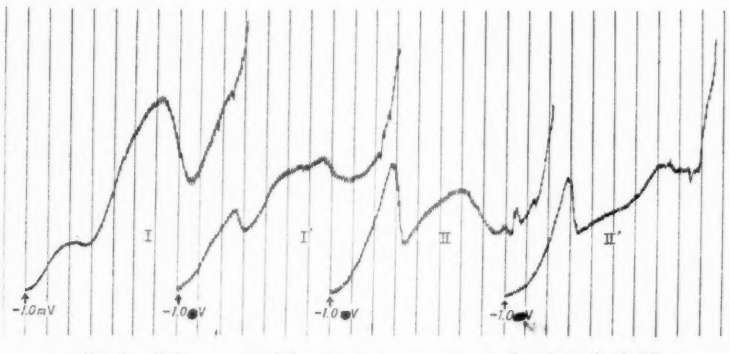


Fig. 5. Polarograms of the cancerous mucoprotein fraction adsorbable to benzoic acid (I), the corresponding normal fraction (II) and their derivatives by treatment with alkaline formaldehyde (See the text p. 373) (I′ and II′).

exchange chromatography

As is shown in Fig. 7, peak D is polarographically most active in the Dowex 1×2 chromatogram, which is the same whether in cancer, in inflammation or in normal. It is noteworthy that, where the polarographically active factor is present, an only low peak is generally demonstrated by

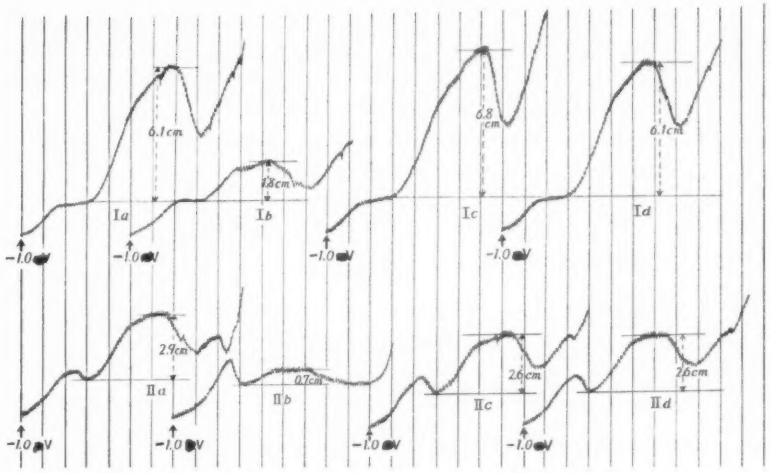


Fig. 6. Comparison in height of the wave at -1.6 V between the mucoprotein fraction adsorbable to benzoic acid and its various derivatives. I*a*: The original cancerous fraction. I*b*: The derivative of I*a* by treatment with NaIO. I*c*: The derivative by treatment with potassium monoiodoacetate. I*d*: The derivative by treatment with diazobenzenesulfonyl chloride and alkali. II*a*: The original normal fraction. II*b*–II*d*: The derivatives of II*a* corresponding to I*b*–I*d*. The polarograms regarding the inflammatory fractions are omitted. The solutions examined were prepared by dissolving 500 γ of substance in 6 cc. of the cobalt buffer as in the case of Fig. 4.

Molisch reaction.

Summary

1) Polarographic behaviours of solutions containing 500–800 γ in 6.0 cc. of the urinary mucoprotein fraction adsorbable to benzoic acid (Kaketa fraction) of cancerous, inflammatory or normal urine were compared with one another. And an index signifying the ratio in height of the wave at about -1.2 V to that at about -1.6 V has been proposed for rough diagnosis whether the individual suffers either cancer or inflammation or is normal, although it can not be availed for strict sense.

2) The indices of urinary KIK-active and KIK-inactive toxohormones of cancerous urine and those of the corresponding normal urine components resemble the corresponding indices of cancerous and normal Kaketa fractions respectively.

3) Polarographic behaviours of various derivatives of Kaketa fractions were also compared with one another. Noticeable finding was that treatment with monoiodoacetate gives rise to wave-heightening in the

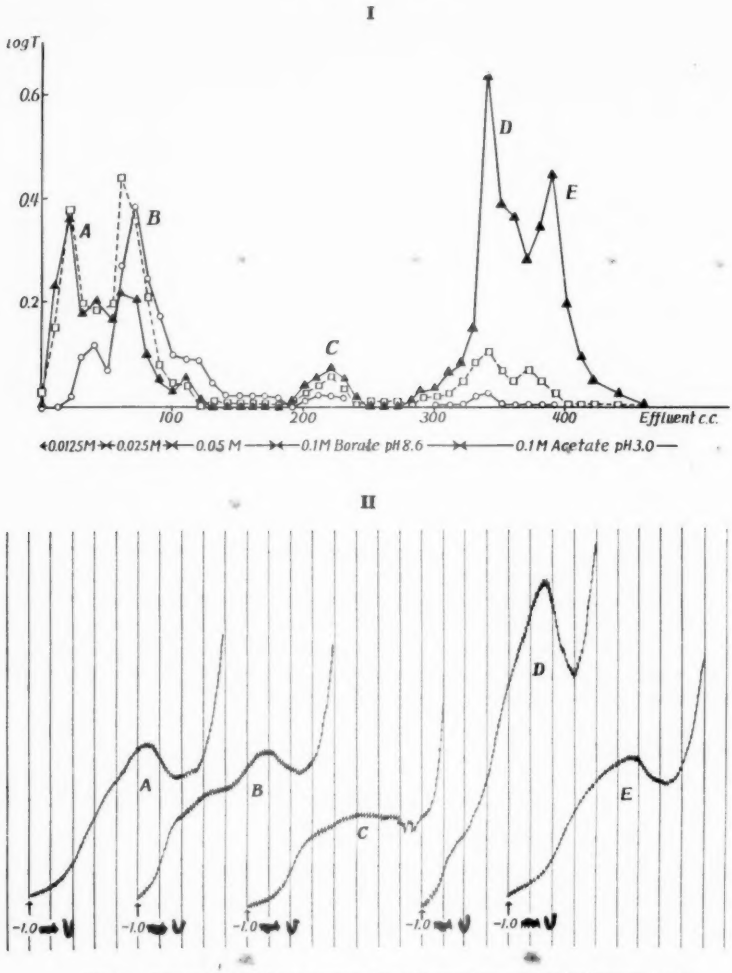


Fig. 7. Separation of the mucoproteins of a cancerous urine by Dowex 1×2 chromatography (**I**) and the polarograms of the separated mucoproteins (**II**). Note the remarkable wave in the polarogram of the component forming peak **D** (See the text p. —). ▲-▲-▲ Ultraviolet absorptions at 280 mμ. □--□--□ Extinctions at 570 mμ after Molisch reaction. ○-○-○ Extinctions at 565 mμ after Ehrlich reaction.

cancerous fraction and to wave-lowering in the inflammatory and normal fractions.

4) Pursuing the polarographically active factors in whole urine by

anion-exchange chromatography, a remarkably active fragment was obtained, though its position in the chromatogram was nearly the same regardless whether the urine was cancerous, inflammatory or normal.

The writers are greatly indebted to Drs. T. Saito and M. Sasaki in Kurokawa Medical Clinic for the kind allowance to use their polarograph at will, whereby this investigation could be carried out, and also for a grant in aid to us from the Ministry of Education through the Grant Committee for Medical, Dental and Pharmaceutical Researches.

References and **Notes**

1) Waldschmidt-Leitz, Z. angew. Chem., 1938, **51**, 324.
2) Meyer-Heck, Z. Krebsforsch., 1939, **49**, 560.
3) Brdička, Collection Czech. Chem., 1933, **5**, 112; 1939, **11**, 614.
4) Brdička, Nature, 1937, **139**, 330, 1020; Acta intern. Verein. f. Krebsbekämpfung, 1938, **3**, 13.
5) Müller & Davis, Amer. J. Med. Sci., 1950, **220**, 298.
6) Winzler & Burk, J. Nat. Canc. Inst., 1943, **4**, 417.
7) Sasai, Polarography, 1957, **5**, 26 (Jap.) (published by the Committee of Polarographic Research, Tokyo).
8) Wada, Gan no rinsho (Clinic of Cancer), 1956, **3**, 764.
9) Wada, Shindan to Chiryo (Diagnosis and Therapeutics), 1957, **44**, 963.
10) Waldschmidt-Leitz & Mayer, Z. physiol. Chem., 1939, **261**, 1; Cf. also 1), f. 326.
11) Sato, Sapporo Medical Journal, 1953, **4**, 432; 1954, **6**, 228.
12) Müller & Davis, J. Biol. Chem., 1945, **159**, 667; Arch. Biochem., 1947, **15**, 39.
13) Mayer, Z. physiol. Chem., 1942, **275**, 16.
14) Winzler, Devor, Mehl & Smyth, J. Clin. Invest., 1948, **27**, 609.
15) Sasai, Saikin no pororagurafi (Review of Modern Polarography) Compiled by Tachi, Published by the Polarographic Research Society, Kyoto Univ., 1951, 50–65.
16) Masamune, Hakomori, Kaketa, Sinohara & Abe, Tohoku J. Exp. Med., 1958, **68**, 63.
17) Kaketa, *ibid.*, 1958, **67**, 319.
18) Masamune, Kawasaki, Abe, Oyama & Yamaguchi, *ibid.*, 1958, **68**, 81.
19) Kawasaki, *ibid.*, 1958, **68**, 119.
20) Masamune, Kawasaki & Abe, *ibid.*, 1958, **68**, 173.
21) Masamune, Kaketa & Abe, *ibid.*, 1958, **68**, 321.
22) Kawasaki, *ibid.*, 1959, **69**, 153.
23) Dickens, Biochem. J., 1933, **27**, 1141.
24) Macleod & Robison, *ibid.*, 1929, **23**, 517.
25) Landsteiner, The specificity of serological reactions, Harvard Univ. Press, Cambridge Mass., 1945.
26) Chromatography of simple proteins especially those of low molecular weight on Dowex 2 was described by Boman and Westlund (Arch. Biochem. Biophys., 1956, **64**, 217; 1957, **70**, 572) and that of mucoproteins of plasma on carboxylated resin by Schmid *et al.* (J. Biol. Chem., 1958, **230**, 853). In view of successful application of Dowex 1×2 column in resolving a mixture of nucleotides, acidic oligosaccharides or polysaccharides, this resin in borate form has been chosen for the present purpose.
27) Dische, Mikrochemie, 1929, **7**, 33.

The Tohoku Journal of Experimental Medicine, Vol. 69, No. 4, 1959

Urinary Mucopolypeptides and Mucoproteins in Cancer

II. An Acid Mucopolypeptide Characteristic of Cancer*

By

Hajime Masamune, Sen-itiroh Hakomori and **Takasi Sugo**
(正宗 一) (箱守 仙一郎) (須郷 昊)

From the Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, February 20, 1959)

In the preceding article[1] we attempted to discriminate the urinary mucoproteins of cancer patients from those of normals and of patients suffering from inflammatory diseases by comparing polarographic waves of them or their various derivatives or by rough fractionation of polarographically active factors of whole urine by the aid of an anion exchanger column. However, our intention to discover any specific change of mucoproteins in cancer was not accomplished, so that efforts were made ever since for much more detailed fractionation of the urinary muco-substances followed by precise chemical analysis of the fractions.

Retrospectively, one of the outstanding features of change in serum or in urine in the case of cancer or inflammation has been the marked elevation of the mucoprotein level, since Lustig[1a] and later on Nilsson[2] and West[3] reported the rise of bound-hexose or-hexosamine level in sera of patients with inflammation or cancer. The finding of Niazi and States[4] in 1948 that cancerous sera react strongly with diphenylamine has been also regarded as due to the increase of mucoproteins, because Werner and Odin[5] remarked in 1952 that "sialoproteins" also give this reaction. The multiplicity of this group of substances in serum has become apparent in last decade by the works of Blix[6], of Rimington,[7][8] of Jayle,[9][10] of Hewitt,[11][12] of Seibert,[13] of Shetlar,[14] of Dische,[15][16] of Schmid,[17][18] of Winzler,[19][20] of our co-workers[21][22] and of many other authors.[23] In 1952 Köiw and Grönwall[24] devised a method for quantitative evaluation of the separated mucoproteins by PAS reaction after paper electrophoresis. It is now a routine procedure in clinical laboratories, although the procedure itself requests technical improvement (Björnesjö[25]). And it has thus become increasingly evident that some definitive relation exists between the quantity of those serum components and

* Read before the 31st Annual Meeting of the Japanese Biochemical Society, Sapporo, July 14–16, 1958. 230th report of Masamune and co-workers' "Biochemical Studies on Carbohydrates."

various pathological processes (Winzler,[26] Greenspan[27], Goa[28], Jayle and Boussier[10]). Nevertheless, many problems still remain to be elucidated about the qualitative-chemical differences of those mucoproteins particularly in connection with systemic pathogenesis of cancer and inflammation.

We are concerned with the chemical qualitative respects of the urinary mucoproteins and particularly with the question whether any specific mucoprotein representing the pathogenic process of canceration exists or not. At first, attempts were made to distinguish the mucoprotein fraction adsorbable to benzoic acid of cancerous, inflammatory and normal urines from one another, whereby a characteristic strongly acidic spot or spots appearing on electrophoresis and a characteristic peak or peaks occurring on ion-exchange chromatography were noticed in the case of cancerous urine. Looking further on for the cancer-specific component among urinary muco-substances, substances were encountered unexpectedly, which are acidic, freely dialyzable through cellophane membrane but not through cellophane membrane coated with celloidin and freely soluble in 90% ethanol or 99% methanol but not in absolute ethanol nor in acetone. Because of smallness of molecule, they are not to be called " mucoproteins " but more appropriately " mucopolypeptides." At least seven such mucopolypeptides were demonstrated in cancerous urine after paper electrophoresis at pH 3.6 and more than ten after anion-exchange chromatography with gradient elution. Increase of one of them has been proved to be fairly specific to cancer, others being detectable also in normal and inflammatory urines. This cancer-specific, strongly acidic mucopolypeptide is named " κ-mucopolypeptide " tentatively. Its quantitative composition and comparison of it with the other acidic mucopolypeptides with respect to structure will be accounted for in a forthcoming paper.

Experimental

Methods

Preparation of the acid mucopolypeptide fraction

A fresh urine is distilled in vacuo to dryness, the residue is dissolved in water of one tenth the original urine volume, and absolute ethanol is added to 90%. The bulky precipitate is centrifuged off and discarded, and the supernatant is distilled to dryness. The residue here is dissolved in water of one fiftieth the original urine volume and precipitated by addition of ethanol to 90%. Spun down. The saturated lead acetate in 90% ethanol is added to the supernatant under agitation until no more precipitate comes out, followed by centrifugation. The centrifugate is freed from lead by stirring in the saturated sodium bicarbonate, and to the clear solution is added again ethanol to 90%. The centrifuged super-

natant fluid given is distilled to dryness, dissolved in methanol of one hundredth the original urine volume and precipitated with ten volumes of acetone. Per 300 cc. of urine of sp. gr. 1.015 were yielded 150 mg. in averages from urine of patients with cancer or inflammation, 80 mg. in averages from that of non-inflammatory patients and from that of normals.

Apparatus for paper electrophoresis

The semi-closed strip method of horizontal type[29] was employed. The closet and the electrode vessels are made with wooden boards coated with a plastic paint. The size of the parts are shown in Fig. 1. The frame to support the paper sheet is made of plastic material as shown in Fig. 2. It can support a 30 cm. wide square of paper and is very convenient not only for two-dimensional electrophoresis of one sample but also for simultaneous one-dimensional electrophoresis of many samples. Power supplier: D. C.; max. load 1000 V, max. current 80 mA. This apparatus was purchased from the Kyowa Institute of Applied Physics, Tokyo.

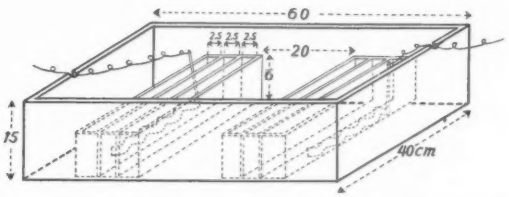


Fig. 1. Cabinet for paper electrophoresis containing four troughs for the buffer solution and two troughs for the electrodes, all made of wooden boards coated with a plastic paint. It is covered with a sheet of glass plate.

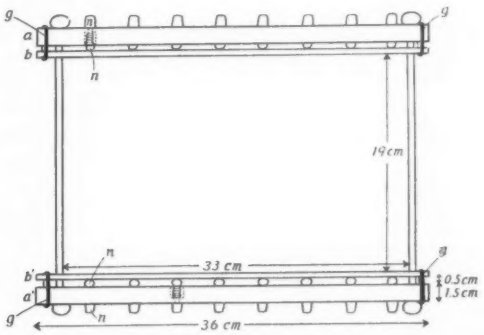


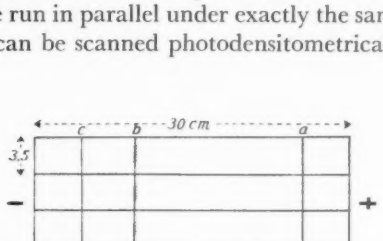
Fig. 2. Frame made of plastic material for holding a paper square. The paper sheet is held tightly between bars b and b' by means of the nobs (n) which are fixed on bars a and a' with stainless wire springs. a and a' are tightly bound with b and b' respectively with gum bands (g).

Buffer solutions used

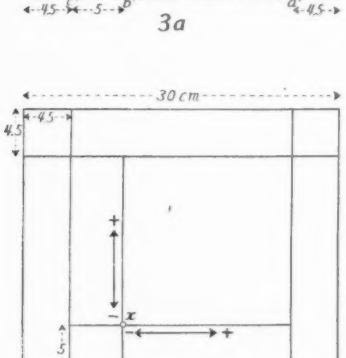
A buffer of pH 3.6, which was a mixture of 1 *M* pyridine and 1 *M* acetic acid in the volume proportion of 1:8, a borate buffer of pH 8.6 composed of 5.5 parts of 0.05 *M* borax (19.105 g. of $Na_2B_4O_7 \cdot 10H_2O$ in 1 *l.* H_2O) and 4.5 parts of 0.2 *M* boric acid (12.36 g. of H_3BO_3 in 1 *l.* H_2O) by volume, and 0.1 *M* borax-HCl buffer of the same pH prepared by dissolving 25.222 g. of $Na_2B_4O_7 \cdot 10H_2O$ in 340 cc. of 0.2 *N* HCl with subsequent addition of water to 1 *l.* were employed for electrophoresis. The latter borate buffer was used also for ion exchange chromatography.

Demonstration of κ-mucopolypeptide among other mucopolypeptides by electrophoresis

In the early stage of cancer when the concentration of κ-mucopolypeptide in the acid mucopolypeptide fraction is low, it is preferable to employ the two-dimensional method because the separation is achieved more sharply. Despite of the incomplete separation, the one-dimensional method has a merit in the point that several specimens including the control can be run in parallel under exactly the same conditions or their component spots can be scanned photodensitometrically.



3a



3b

Fig. 3. Size of the parts in the paper sheets for one-dimensional (**3a**) and two-dimensional (**3b**) electrophoreses.

Electrophoresis

Toyo Roshi filter paper No. 51 is used throughout the experiment. Pencil lines are drawn on the paper as shown in Fig. 3, and 0.03 cc. of a 50% solution of the above fraction is put on the *b*–*b'* line in paper **3a** or double the volume of the solution on point *x* in paper **3b**. The sheet dried is sprayed evenly with the acetic acid-pyridine buffer of pH 3.6, plenty enough to avoid centripetal hydrodynamic flow from both the buffer troughs, fixed on the frame and settled between the two buffer troughs, so as line *c*–*c'* faces cathode and line *a*–*a'* faces anode in the case of one-dimensional electrophoresis. The two buffer troughs are connected with each other and the outer trough with the electrode vessel by means of a paper strip or agar bridges, and the levels of the pair of the inner buffer troughs are equilibrated by a tubing. The nonpolar electrodes are constructed beforehand in 1% KCl–Ag-AgCl system. A drop of acid fuchsin is put beside each of the sample spots. Electricity is flowed at constant 200 V or at constant current 0.35 mA per cm. width of paper. Duration of test 24 hours. Temperature below 10°C (In summer, the cabinet is cooled with chunks of ice placed on the bottom). Then, the paper is dried by a drier. In the case of two-dimensional electrophoresis, the dried sheet is further sprayed with the borax-boric acid buffer of pH 8.6 and settled in the assemblage to process the secondary run at 400 V of electricity for 2 hours.

After thorough drying of the paper by mild heating, the spots are indicated by an appropriate reaction. As regards that reaction were examined such reagents as $NaIO_4$-$AgNO_3$-NaOH[30], *p*-dimethylaminobenzaldehyde[31], HIO_4-Shiff,[24] ninhydrin, aniline phthalate, Toennies' platinic iodide reagent for SH-group,[32] iodine vapor (Kent[33]), Cl_2-ClO_2-benzidine (Raydon[34], Reindel and Hoppe[35]), α-naphthol-H_3PO_4[36] and HIO_4-$KMnO_4$ (or $NaIO_4$-$KMnO_4$)[37][38]. The spots were also revealed under an ultraviolet lamp. The last two of the reagents were found satisfactory contrary to the others, by which the tests are time-consuming and tedeous or too nonspecific.

a) HIO_4-$KMnO_4$ method. Lemieux and Bauer[37] and later on Wolfrom and Miller[38] employed this reagent for the test of nonreducing sugars, but the reaction was let occur in an alkaline medium, whereby amino acids, peptides or even various cations react though faintly so that the method has been improved to avoid nonspecific reaction by conducting the assay in a slightly acidic medium as follows :— The paper is first sprayed with 2% HIO_4 (Merck New York), and after standing at room temperature for 8 to 10 minutes, again sprayed but with 2% $KMnO_4$ in water (2% $KMnO_4$ in 1% acetic acid when an alkaline buffer is used in electrophoresis). In 10–15 minutes, spots get colored yellow to brown against a purple background. The paper is washed with water well to remove

the purple $KMnO_4$ colour as far as possible and air-dried. Brown spots indicate the presence of periodate-reactive substances. The specificity of the reaction was checked with amino acids, amines, urea and creatinine, all of which proved inert.

The reaction can occur after ninhydrin reaction without altering its sensitivity. In quantitative densitometry by means of a Beckman scanning meter, the time of reaction and the temperature should be controlled. Five minutes oxidation with periodate at 10–15°C, ten minutes exposure to permangate at the same temperature and 10 minutes washing with tap water in turn meet the purpose.

b) α-Naphthol-H_3PO_4 method

The paper on which samples have migrated electrophoretically is sprayed with a mixture of 9 parts of 1% α-naphthol in ethanol and 1 part of concentrated orthophosphoric acid and heated over an oven for about 5 minutes at 80°C, until the coloration of spots reaches maximum. Overheating should be evaided. Pink, blue, violet and green spots are shown against a white background.

Assemblage for simultaneous ion exchange chromatography of three samples by gradient elution

Tiselius principle of gradient elution[39] allowing an exponential increase of eluting force was applied. In order to carry out chromatography of three different samples (cancerous, normal and inflammatory) under exactly the same conditions, the assemblage in Fig. 4 was used which can maintain the dropping rates of the eluate uniform and constant. A hypodermic syringe, through which a stainless stylet is run, is connected with the tip of a column to regulate the drop rate by pushing in or pulling out the stylet.

Preparation of the column

Commercial Dowex 1×2 in chloride form of mesh 200–400 is set free by washing with 2 *N* NaOH and washed with water and 2 *N* HCl in turn. The operation is repeated twice. The resulting Dowex 1×2 in chloride form was washed with changes of the 0.1 *M* borax-HCl buffer of pH 8.6 until no more occurrence of pH change of the buffer, poured into a 1.3 cm. wide chromatography tube to make a column of 15 cm. height and washed with the borate buffer diluted 16 times (0.00625 *M*).

Column chromatography

300 mg. of the acid mucopolypeptide fraction of cancerous, inflammatory and normal urines are dissolved each in 1–2 cc. of water and added onto three columns separately. The chromatography tubes are combined with the assemblage shown in Fig. 4, in which mixing bottle *b* containing 750 cc. of the 0.00625 *M* borax-HCl buffer of pH 8.6 is surmounted with a tubelet connecting it with reservoir *a* containing 1200 cc. of the 0.1 *M*

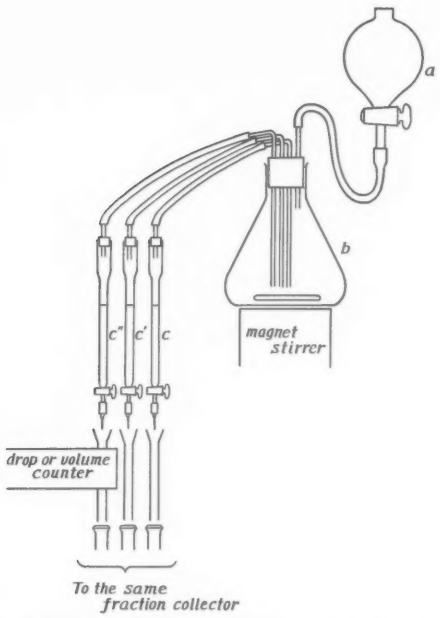


Fig. 4. Assemblage for simultaneous chromatography of three samples. Three tubings connect three different chromatographic tubes (*c*–*c''*) with a 1 *l.* mixing bottle (*b*) which is surmounted with a dropping funnel (*a*).

borax-HCl buffer of the same pH value. A magnetic stirrer is set beneath the mixing bottle. The dropping rate is so adjusted as one drop falls in 8 to 10 seconds. When the 0.1 *M* borate buffer in the reservoir is exhausted, 900 cc. of 0.5 *M* acetic acid is replaced for continuous elution. Subsequently successive elutions with 0.5 *M* formic acid and 0.33 *M* hydrochloric acid were usually processed in a similar manner. The effluent is collected in 10 cc. fractions.

1 cc. aliquots of each fraction are analyzed by a quantitative Molisch reaction according to Dische[40] (Extinctions at 570 mμ are measured after the reaction) and another 3 cc. aliquots are examined regarding the ultraviolet absorption at 280 mμ.

Results with Discussion

One-dimensional electrophoretic patterns consist of at least seven spots in case of cancerous urine and five to six spots in cases of normal and noncancerous pathological urines (See Fig. 5 and 6). The spots are designated A, B, C, D, E, F and G in the sequence from the slowest to the fastest. Sometimes spot H is observed besides, irrespective of the kind

HEALTH CENTER LIBRARY

Fig. 5. Paper electrophoretic paptterns of the mucopolypeptide fraction of urines of patients with gastric cancer at the early stage (I), gastric cancer with liver metastasis (II), hepatoma (III), gastric ulcer (V), tuberculosis of lung (VI) or advanced gastric cancer with ascites (VII), and of a pregnant (fourth mouth) women (IV). The patterns were indicated by periodate-permanganate method (Oxidize with KIO_4, spray with $KMnO_4$ and wash with water). Note appearance of K-mucopolypeptide (area E) in case of malign tumors (I, II, III and VII) but not in other diseases nor in pregnancy. Regarding conditions for the electrophoresis, see the text.

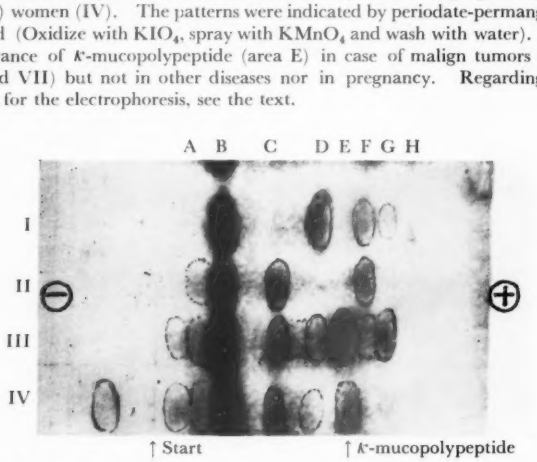


Fig. 6. Paper electrophoretic patterns of the mucopolypeptide fraction of urines of patients with gastric ulcer (I), tuberculosis of lung (II), gastric cancer (III) or leucemia (IV). Note the occurrence of spots at positions corresponding to K-mucopolypeptide (E area) in III and IV.

of disease. The spots react with $NaIO_4$-$KMnO_4$ and are all colored brown but in the order of B, D, E, F, C, A and G in strength, the last spot being the weakest. However, their reactivities with the α-naphthol-

phosphoric acid reagent are conspicuously different. Spot B gets colored brownish or greenish purple, A and C faintly red, and G and E intensely purple, while D remains almost uncolored.

As B and C are reactive towards *p*-dimethylaminobenzaldehyde and Toennies' SH-reagent, they contain sialic acid and an amino acid grouping possessing the SH-radical in the molecule, in other words, they belong to a cathegory of "sialoproteins" or "neutral glucidamins", resembling α_1- or α_2- acid glycoprotein in Schmid's or Winzler's terminology. In contrast to those substances, that forming spot E is quite unique in composition, because it gives spots at the positions corresponding to glucosamine, a methylpentose, an unidentified reducing aminosugar and such amino acids as aspartic and glutamic acids, glycine, serine, alanine and so on but none of cyclic amino acids nor hexone bases, when paper-chromatographed after hydrolysis, and also shows the positive reaction for hydrolyzable sulfur. Quantitative estimation of the zones of the hydrolysate after electrophoresis on "Geon"-polyvinyl resin[41] or photodensitometric scanning of the spots after paper-electrophoresis by means of a Beckman photo-scanning meter or even by comparison of the spots by the naked eye revealed in case of cancer i) appearance or marked increase of spot E which is reactive toward periodate-permanganate and also towards α-naphthol-phosphoric acid, and ii) increase of spot G which is reactive towards periodate-permanganate but not towards α-naphthol-phosphoric acid. A unique spot migrating towards cathode in case of monocytic leucemia is also remarkable. In tuberculosis marked increase of spot F is noticed. Increase or appearance of spot E has never been experienced in any diseases other than cancer. It is more pronounced at the advanced stage than at the early stage of cancer. The distance of the spot (center), which owes to κ-mucopolypeptide, from the start varies from 8 to 10 cm. The one-dimensional electrophoresis patterns represented photodensitometrically by means of a Beckman scanning meter are shown in Fig. 7. It is of great interest that different spots increase or decrease according to the kind of disease.

Two-dimensional paper electrophoresis can resolve the acid mucopolypeptide fraction into at least eight components much more sharply than the one-dimensional, although it requests more time and skill. Fig. 8 shows examples of the patterns.

A number of peaks are well separated by ion exchange chromatography as illustrated in Fig. 9, 10 and 11. Of the fast occurring peaks, one which is especially high in cancer might correspond to spot B in the electrophoresis pattern, and a peaks pecific to cancer which coincides with the spot of κ-mucopolypeptide after electrophoresis is demonstrated soon after commenecment of the acetic acid elution. The latter peak is Molisch-positive but devoid of ultraviolet absorptivity. Thus, besides the peaks common

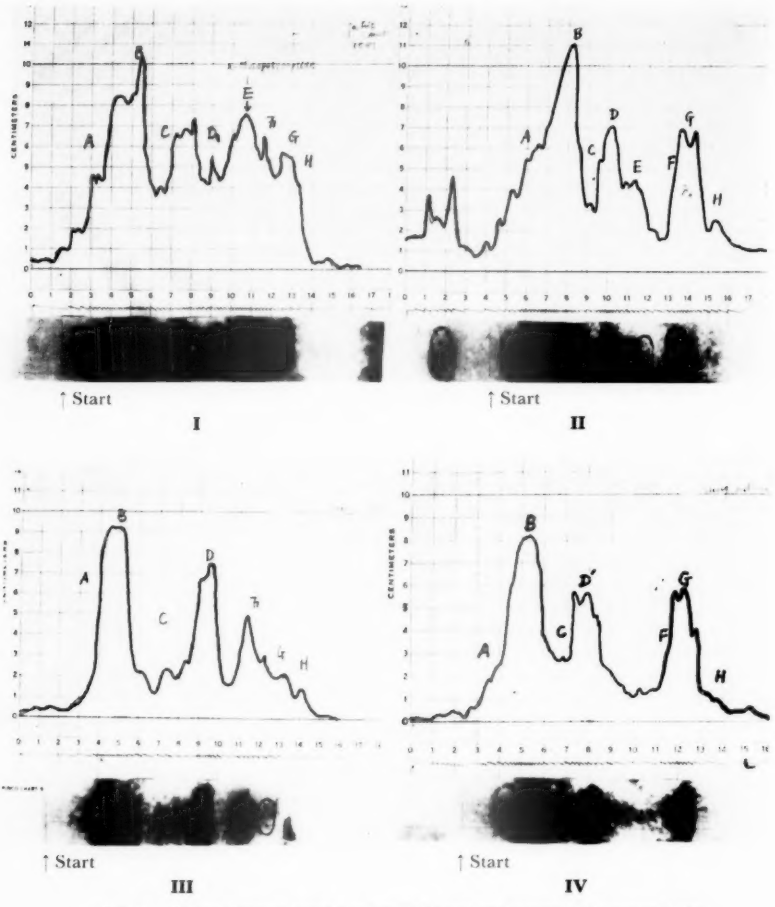
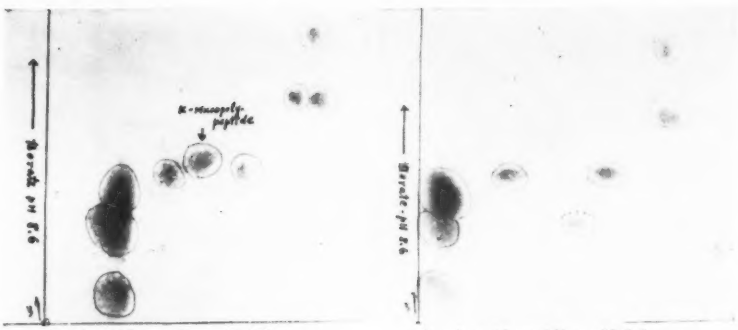


Fig. 7. Paper electrophoretic patterns photodensitometrically scanned immediately after periodate-permanganate staining of the urinary mucopolypeptides of various diseases. Note appearance of the κ-mucopolypeptide peak at E between D and F in cases of malign tumor. **I**: Gastric cancer with metastasis in liver. **II**: Monocytic leucemia. **III**: Gastric ulcer. **IV**: Tuberculosis of lung.

to normal and pathological urines, a peak or peaks which reflect particular types of disease are also discerned in the ion-exchange chromatograms. The technique requires a long time, but the patterns obtained are quite characteristic and decisive for diagnosis. The procedure to get such patterns may be called " muco-urinography ", and the patterns " muco-urinograms ".



Acetic acid-pyridine pH 3.6 ⟶ Gastric cancer

Acetic acid-pyridine pH 3.6 ⟶ Pregnancy

Fig. 8. Two-dimensional electrophoretic patterns of the mucopolypeptide fraction of urines of a patient with gastric cancer (left) and of a pregnant woman (right). The first run was effected in 0.1 *M* pyridine-acetic acid buffer of pH 3.6 and the second in 0.1 *M* borax-boric acid buffer of pH 8.6. The direction of the anode is coincident with the arrow mark. Regarding conditions else of electrophoresis see the text. The spots were oxidised with $NaIO_4$ in 1% acetic acid for 5 minutes at 10°C at first and then exposed to 2% potassium permanganate for 10 minutes at 10°C followed by washing with water for 10 minutes. Note clear-cut separation of the components from one another in the both patterns and marked occurrence of κ-mucopolypeptide in the left.

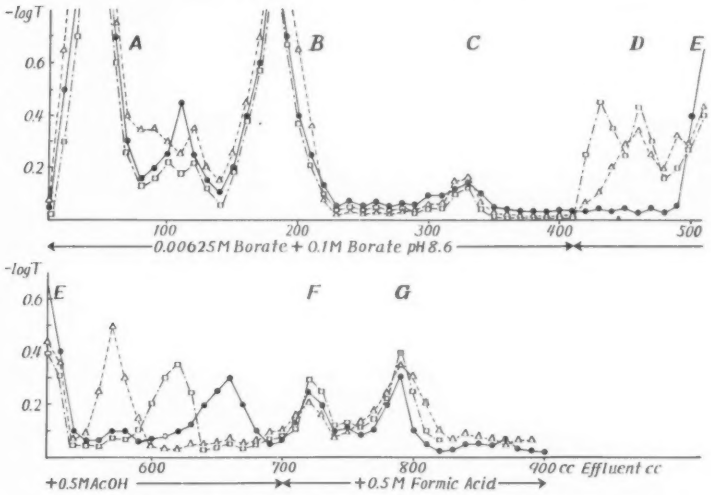


Fig. 9. Dowex 1×2 chromatograms of the mucopolypeptide fraction of urines from a patient with gastric cancer (△ - - △ - - △), a patient with monocytic leucemia (□ - - □ - - - □) and a normal (● ● ●). The extinctions at 570 mμ of the fractions were measured after quantitative Molisch reaction. Notice that a peak occurs at the first stage of the acetic acid elution in the cases of cancer and leucemia but not in the case of normal.

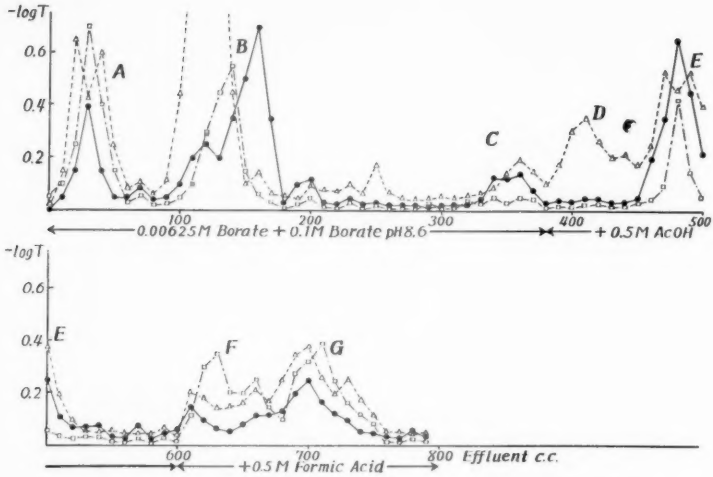


Fig. 10. Dowex 1×2 chromatograms of the mucopolypeptide fraction of urines from a patient with gastric cancer (△--△--△), a patient with gastric ulcer (●—●—●) and a patient with hepatitis acuta (□---□---□). The extinctions at 570 mμ were measured after quantitative Molisch reaction of the fractions. Note the occurrence of a peak at the first stage of the acetic acid elution only in the case of cancer.

It is strongly suggested from electrophoretic patterns and muco-urinograms that the special mucopolypeptides that increase or appear reflect responsible pathogenic processes inflammation, degeneration, tissue proliferation, systemic alarming reaction, malign growth etc. Prospectively thinking, the muco-urinographic analysis would make an epoch not only for better understanding of urinary colloids but also for fundamental utilization of them in prognosis and diagnosis of various pathological processes.

In Table I are summarized the results of assay for κ-mucopolypeptide compared with those of clinical diagnosis.

Summary

1. A cancer-specific acid mucopolypeptide was found among other urinary muco-polypeptides and designated "κ-mucopolypeptide".

2. Methods for detecting it in cancerous urines are described.

The writers express their hearty gratitude to the Ministry of Education for a grant in aid to us trough the Grant Committee for Medical, Dental and Pharmaceutical Researches.

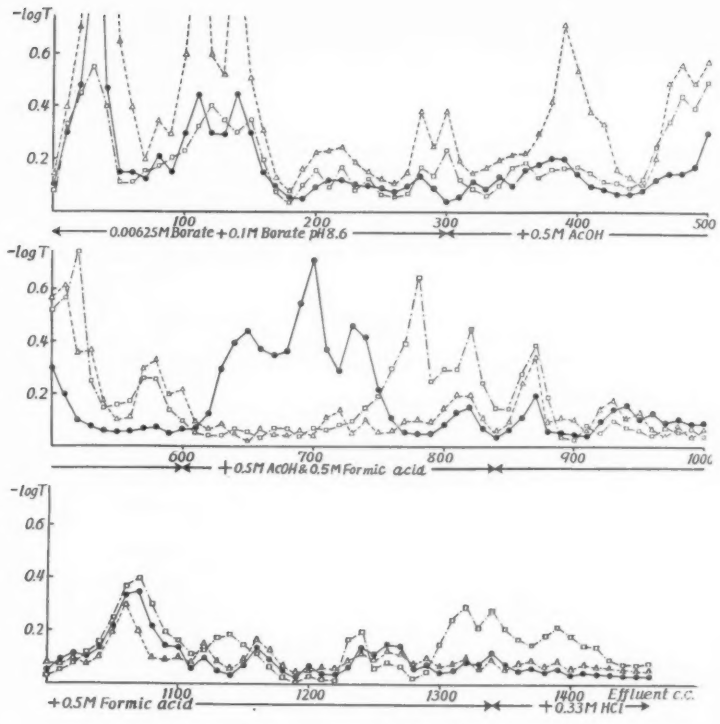


Fig. 11. Dowex 1×2 chromatograms of the urinary mucopolypeptide fraction of patients with gastric cancer (△- - △ - - △), gastric ulcer (●–●–●) or tuberculosis of lung (□ - - - □ - - - □). The gradient elution here was made by inserting a mixture of equal parts of 1 *M* acetic acid and 1 *M* formic acid between 0.5 *M* acetic acid and 0.5 *M* formic acid, and the extinctions at 575 mμ of the fractions after quantitative Molisch reaction were measured. Note the occurrence of a peak at the first stage of the acetic acid elution only in the case of cancer.

References and Notes

1) Masamune, Hakomori, Kaketa & Sugo, Tohoku J. Exp. Med., this volume p. 371.
1a) Lustig & Langer, Biochem. Z., 1931, **242**, 320.
2) Nilsson, *ibid.*, 1937, **291**, 254.
3) West & Clarke, J. Clin. Invest., 1938, **17**, 173.
4) Niazi & States, Cancer Res., 1948, **8**, 653.
5) Werner & Odin, Acta. soc. med. Upsal., 1952, **57**, 227, 230.
6) Blix, Tiselius & Svensson, Biochem. J., 1941, **37**, 485.
7) Rimington, Ergebn. Physiol., 1933, **35**, 712.
8) Rimington, Bioch. J., 1940, **34**, 931.

TABLE I

Diagnosis of Cancer by Assay for κ-Mucopolypeptide in Urine

Clinical diagnosis		Treatment received	Occurrence of κ-mucopoly-peptide
Gastric cancer	Early stage, no metastasis		±
Gastric cancer	Early stage, no metastasis		+
Gastric cancer	Metastasis in liver	Laparotomy, chemo-therapy	+
Gastric cancer	Metastasis in liver with perito-nitis carcinomatosa	Chemotherapy	+
Gastric cancer	Metastasis in liver, with Vir-chow noduli and peritonitis car-cinomatosa	Chemotherapy	+
Gastric cancer	Postoperative recurrence, metas-tasis in liver	Chemotherapy	+
Gastric cancer	Metastasis in liver with perito-nitis carcinomatosa	Chemotherapy	+
Esophagus cancer	Metastasis in liver and media-stinal cavity	Chemotherapy	+
Uterus cancer	Origin at portio vaginalis, 4th grade	x-Ray and radium irradiation	+
Uterus cancer	Origin at portio vaginalis, cir-cumscriptive	x-Ray and radium irradiation	−
Vaginal cancer	Origin at labium minor, no metastasis	Radium irradiation	−
Breast cancer	Early stage	x-Ray irradiation	−
Monocytic leucemia		Chemotherapy	+
Hepatoma	Primary	Chemotherapy	+
Mediastinal tumor		x-Ray irradiation	±
Chorionepithelioma malignum	Postoperative recurrence, with lung metastasis		±
Hodgkin's disease		Chemotherapy	−
Tuberculosis of lung	Productive	Chemotherapy	−
Tuberculosis of lung	Exudative	Chemotherapy	−
Pregnancy	4th month		−
Pregnancy	10th month		−
Pregnancy	10th month		±
Puerperal fever		Chemotheropy	−
Rheumatic fever			−
Normal			−
Normal			−
Gastric ulcer	Niche remarked on x ray exa-mination		−
Gastric ulcer	"		−
Gastric ulcer	"		−

9) Jayle & Abdellatif, Bull. soc. chim. biol., Paris, 1946, **28**, 80.
10) Jayle & Boussier, Expos. ann. biochem. med., 1955, **17**, 157.
11) Hewitt, Biochem. J., 1937, **31**, 360.
12) Hewitt, *ibid.*, 1938, **32**, 1554.
13) Seibert, Pfaff & Seibert, Arch. Biochem., 1948, **18**, 279.
14) Shetlar, Shetlar, Richmond & Everett, Cancer Res., 1950, **10**, 681.
15) Dische, Biochem. Z., 1928, **201**, 74.
16) Dische & Osnos, Fed. Proc., 1952, **11**, 202.
17) Schmid, J. Amer. Chem. Soc., 1953, **75**, 60.
18) Schmid, *ibid.*, 1955, **77**, 742.
19) Weimer, Mehl & Winzler, J. Biol. Chem., 1950, **185**, 561.
20) Winzler, Devor, Mehl & Smyth, J. Clin. Invest., 1948, **27**, 609.
21) Ozaki, J. Biochem. (Japan), 1936, **24**, 73.
22) Osaki & Yosizawa, Tohoku J. Exp. Med., 1949, **51**, 57.
23) Regarding reviews of those accounts see Winzler, Advances in cancer research, Academic Press, New York, 1953, **1**, 503; Methods of biochemical analysis, Interscience Publishers, New York and London, 1955, **2**, 279.
24) Köiw & Grönwall, Scand. J. Clin. & Lab. Invest., 1952, **4**, 224.
25) Björnesjö, *ibid.*, 1955, **7**, 153.
26) Winzler, Chemistry & biology of mucopolysaccharides, Charchill, London, 1958, p. 245.
27) Greenspan, Arch. Intern. Med., 1954, **93**, 803.
28) Goa, Scand. J. Clin. & Lab. Invest., 1955, Suppl. 22.
29) Block, Durrum & Zweig, Paper chromatography & paper electrophoresis, Academic Press Inc., New York, 1955, p. 349 (Table I).
30) After oxidation with $NaIO_4$, the paper was treated by Travelyan and others' procedure (Trevelyan, Procter & Harrison, Nature, 1950, **166**, 444). Mucoproteins can be revealed as brown to black spots on a white background, but the specificity of this indication method is very low.
31) Dissolve 0.8 g. of Ehrlich aldehyde reagent in a mixture of 30 cc. of conc. HCl and 30 cc. of abs. ethanol. Sprayed with the reagent and warmed gently, sialoprotein spots get colored purple.
32) Toennies & Kolb, Anal. Chem., 1951, **23**, 823–826.
33) Greenway, Kent & Whitehouse, Research, 1953, **6**, Suppl. No. 1, 6; Cited in C. A. 1954, **48**, 1203.
34) Raydon & Smith, Nature, 1952, **169**, 922.
35) Reindel & Hoppe, Naturwissenschaften, 1953, **40**, 221.
36) Rachinskii & Knyazyatova, Doklady Aka. Nauk S.S.S.R., 1952, **85**, 1119; Cited in C. A., 1955, **47**, 448 and by Block *et al.* (29)), p. 136.
37) Lemieux & Bauer, Anal. Chem., 1954, **26**, 920.
38) Wolfrom & Miller, *ibid.*, 1956, **28**, 1037.
39) Alm, Williams & Tiselius, Acta chem. Scand., 1952, **6**, 826; Hagdahl, Williams & Tiselius, Arkiv. kemi, 1952, **4**, 193; Cited in Lederer & Lederer, Chromatography, Elsevier Publ., Amsterdam, London, New York & Princeton, 1957, p. 43.
40) Dische, Mikrochemie, 1929, **7**, 33.
41) Geon-polyvinyl resin of mesh 200–400, Geon Co., Cleaveland, Ohio was used.

The Tohoku Journal of Experimental Medicine, Vol. 69, No. 4, 1959

An Autopsied Case of a Newborn with Asplenia, Accessory Liver, Malformation of the Heart and the Major Blood Vessels and Agenesis of the Corpus Callosum in Complication

By

Akira Kajita
(梶田 昭)

From the Pathological Laboratory of Prof. K. Akazaki, Faculty of Medicine, Tohoku University, Sendai

and

Chisato Yamada and **Haruo Asano**
(山田千里) (朝野晴雄)

From the Department of Obstetrics and Gynecology, Sendai National Hospital, Sendai

(Received for publication, March 4, 1959)

Recently, the present authors had the experience of examining the subject case in autopsy. These anomalies are rare enough individually, and it is surely worthy of special attention that such anomalies should be found in coexistence in one child. So, we will hereunder report the autopsy findings on it in summary and make some relevant discussions.

Report on the Case

Extract from the clinical records : The case (a girl) was born by normal full-term delivery at 7.25 a.m. on Jan. 2, 1959 (termination was Jan. 3), as the first child of healthy parents. At birth she measured 50 cm. in length and 2,940 g. in weight and was seemingly well developed. Soon, however a severe cyanosis set in and the patient was treated using Oxygen-Air Pressure Lock (Bloxosom) and injection of cardiotonics was made. But light cyanosis had been observed in spite of these treatments. Everytime the patient was taken out of Lock for the purpose of enforced nutrition through catheterisation, increase of cyanosis was observed. Many kinds of treatment such as cardiotonics, hormone therapy, nutritional therapy was proved to be ineffective, she died at 4.40 a.m. on Jan. 6.

Outstanding patho-anatomical and histological findings : 1) Complete defect of the interventricular septum presenting the heart picture of cor biatriatum

triloculare, and atresia of the pulmonary artery trunk. The aorta having three valve leaflets formed the pseudotruncus, blood flowing thence, via the greatly dilated ductus arteriosus to the branches of the pulmonary arteries right and left. The formaen ovale was already closed. Histologically examined, the lungs showed very prominent dilation and congestion in the bronchial vascular beds, suggesting that the deficiency in pulmonary blood flow through the pulmonary arteries was compensated by that via the bronchial blood vessels. 2) Asplenia and absence of the splenic vessels. Upon following up the vessels traversing the normal pathways of the splenic artery and vein leftwards along the pancreas, these were found to taper off rapidly and ending in the pancreas and its capsular connective tissue. 3) Accessory liver found on the cranial pole of the left adrenal gland, in the form of a flat tissue mass, encased in a collagenous capsule and containing hepatic cell cords and Glisson's capsule (Fig. 1). In some parts, some aberrant adrenocortical (outer zone) cells were found emigrated beyond the adrenal capsule and forming islets in the tissue of the accessory liver (Fig. 2). 4) Agenesis of a large part of the corpus callosum (Fig. 3). The rostrum and the genu of the corpus were attenuated but still present, but in the coronal section through the posterior part of the frontal lobe and the parietal lobe, we found only bare vestiges of the corpus on both the hemispheres and completely disjoined into two parts. The septum pellucidium was nearly normal. The anterior commissure could not be detected marcroscopically. Thus, agenesis was observed in the two important white matters uniting the two cerebral hemispheres. No macroscopic anomaly was found in the other parts of the brain.

Comment and Conclusion

In 1927, Lubarsch[1] collected 30 cases (of which 11 cases had also malformations in their heart and major vessels) of reported asplenia of all age-classes. In 1953, Okudaira[2] reported on a case of asplenia complicated with cor biloculare and accessory liver, a girl of 19 months, for the first time in Japan.

Accessory liver found in various parts of the abdominal cavity have been described by Hanśer[3]. Among these, the case (reported by Krauss) with a fist-size lump at the site of the right adrenal gland and subjected to surgical operation is of special interest. Attempts have been made to explain asplenia and accessory liver in connection with anomalies in the major blood vessels (e.g. by Tarozzi[4]), but in the preceding case, such an explanation can hardly serve.

It is well known that in some cases a part of the adrenal tissue are found to form in the extracapsular adipose tissues or loose connective tissues, nodules or noncapsulated cell masses. In our case, we found some

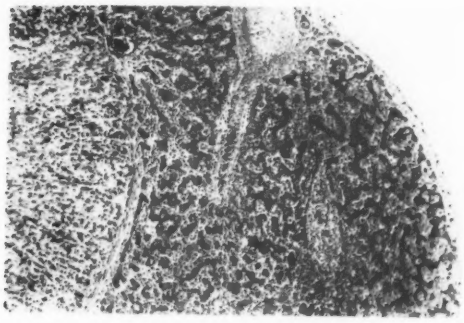


Fig. 1. Accessory liver (on right) on the cranial pole of the adrenal gland (H. E. staining, ×35).

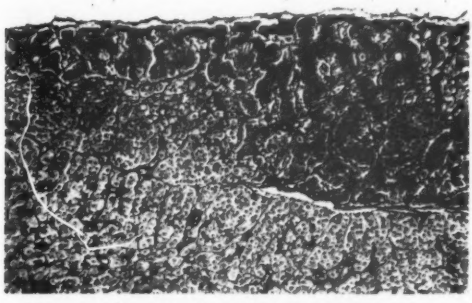


Fig. 2. Aberrant adreno-cortical cellmasses emigrated beyond the adrenal capsule and forming islets in the tissue of accessory liver (Silver impregnation, ×100).

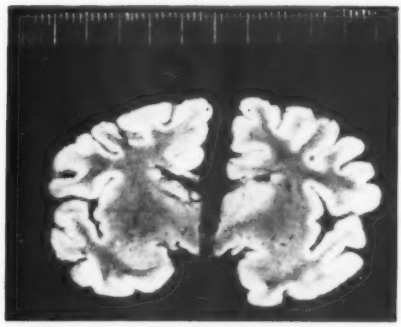


Fig. 3. Transverse section of the brain showing vestiges of the corpus callosum in the both cerebral hemispheres.

adreno-cortical tissues immigrated islet-wise into the accessory liver—a

finding apparently understandable from such a characteristic of the adrenal tissues.

Cases of defective human corpus callosum have been reported on more than 100 occasions (Ogawa[5]). Phylogenetically speaking, such cases may be said to have brains of low-class mammals (acallosal mammals). On the other hand, the anterior commissure consists of commissural fibres of the rhinencephalon and is of much earlier origin.

Ontogenetically, it has been confirmed that the corpus callosum comes into formation during the third-fourth month of fetal life[6], and it may be presumed that the partial agenesis of the corpus in our case had been caused by a teratogenic factor active in such a stage. On the other hand, anomalies in the heart is said to be determined within the 8th week of intrauterine life (Potter[7]). Therefore, such combinations of malformations in various parts as found in our case are apparently not to be explained by a simultaneous action of stresses in the embryological process.

References

1) Lubarsch, Henke-Lubarsch' Hdb. spez. Path., Springer, Berlin, I/2, 1927.
2) Okudaira, Trans. soc. path. Jap., 1953, **42**, 195.
3) Hanser, Henke-Lubarsch' Hdb. spez. Path., Springer, Berlin, V/1, 1930.
4) Tarozzi, Zbl. Path., 1905, **16**, 712.
5) Ogawa, Anatomy of the brain (jap.). Nanzando, Tokyo, 1953.
6) Cass & Reeves, Arch. Surg., 1939, **39**, 667.
7) Potter, Pathology of the fetus and the newborn, The Year Book Publishers, Chicago, 1952.

VOLVO 440/460 (87-96)

AUTOMOTIVE REPAIR MANUAL

XX